Design and Characterization of an Intraoperatively Loaded Protein Delivery Device for the Treatment of Open Tibial Fractures

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Abstract

Design and Characterization of an Intraoperatively Loaded Protein Delivery Device for Treatment of Open Tibial Fractures

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The FDA approved INFUSE Bone Graft is the standard of care for the therapeutic delivery of BMP-2 in open tibial fractures stabilized via intramedullary nailing. Though the device is considered clinically effective, several serious and potentially preventable side effects induced by the Bone Graft's initial burst release characteristics, such as massive swelling, cystic bone growth, and ectopic bone growth have negatively impacted the lives of countless patients. Based on analysis of recent studies involving BMP-2 delivery technologies, it was determined that a lower dose, sustained release of BMP-2, averaging 1.37 to 25.7 µg per day over at the duration of at least 2-4 weeks, absent a burst release, would dramatically decrease the chances of adverse side effects while still providing the therapeutic benefits of BMP-2. Several new technologies have been developed that provide similar release profiles to the abovementioned, and they have shown much promise in several *in vivo* and *in vitro* studies. However, the majority of those technologies rely on pre-encapsulation of the BMP-2 and harsh polymer processing techniques that facilitate denaturation of the protein. Intraoperative loading circumvents this shortcoming, since the BMP-2 is stored in its stable, lyophilized form until needed.

Over the course of this design project, a novel, biodegradable protein delivery device was developed in an attempt to combine the advantages of intraoperative loading with a more clinically relevant delivery profile for BMP-2.

The proposed design consisted of a two component system comprising a Polycaprolactone : PEG 400 composite outer pouch with an internal, fibrous sodium alginate sponge. The system was designed for intraoperative loading using a technique similar to the INFUSE Bone Graft, where a reconstituted model protein solution (bovine serum albumin) is injected into the PCL: PEG 400 pouch containing an alginate sponge. After a brief 5 minute wait, a 10 % w/v $CaCl_2$ solution is injected into the pouch, causing crosslinking of the swelled alginate and encapsulation of the protein. The pouch is then tightly sealed with a solvent bonding approach using ethyl acetate. This pouch design allows for successful loading with reconstituted protein solution within 20 minutes.

The release characteristics of the device, as well as alternative preparation methods and their effect on the pouch release rate were investigated through dissolution testing in simulated physiological conditions (PBS, pH 7.4 & 37ºC) and the Bradford-Coomassie assay. Several pouch formulations were assessed, and three were capable of providing sustained release of BSA in the desired 1.37-25.7 µg range over 14 days, with no burst release. It was determined that modifying the ratio of PCL to PEG 400 would cause a predictable change in the release profile of the pouches, in that the higher the PCL : PEG 400 ratio, the slower the release.

In addition to the protein delivery aspects, the material degradation and surface morphologies of the pouch were monitored over the desired dissolution period to understand this novel technology to the degree that future modifications to the design could be conducted if necessary. The polymer degradation rate of the pouch was assessed with inherent viscosity testing and GPC. The results of these tests show a minimal decrease in IV, Mn, and Mw over 16.4 days, suggesting that no substantial

degradation occurs in the pouch material during the delivery period. SEM imaging of the pouch material over the delivery period combined with the previous studies suggest that when the material is placed in aqueous media, the PEG 400 will dissolve out rapidly. A mass change experiment was attempted to quantify the amount of PEG 400 lost over time once placed in aqueous media, which showed that 90.4 % of the PEG 400 was lost within 1 minute, 98.3 % within 1 hour, and ~100% of the total PEG 400 content within 5 hours of submersion. The removal of PEG 400 from the pouch left behind a porous PCL material exhibiting distributed pores approximately 2-5 μm in diameter. This network acted as a rate controlling membrane, limiting mass transport into and out of the pouch, allowing for sustained release of the encapsulated protein from the sodium alginate via diffusion, while preventing a burst release.

A preliminary BSA specific competitive ELISA was conducted to determine if the model protein could be encapsulated and released from the delivery device over a two week period without damage to its binding sites specific to the ELISA. Successful binding would therefore suggest that the overall tertiary structure of the BSA was uncompromised. The results of the ELISA showed consistent, sustained release from three different pouch formulations, thereby verifying that a complex protein could be successfully loaded into the device and delivered in a controlled, predictable manner, with no adverse effects on the protein structure.

Bioactivity testing with a BMP-2 specific ELISA and the Alkaline Phosphatase assay (ALP) with C2C12 cells was planned, but due to time and cost constraints, the test was postponed. Nevertheless, the previous results have been quite encouraging, and low bioactivity from BMP-2 released from the device was not expected.

Chapter 1: Introduction

1.1 Bone Morphogenetic Protein-2 history and Indications for Use

Bone morphogenetic proteins (BMPs) consist of 20 growth factors belonging to the TGF- β superfamily of proteins. Initial interest in BMPs began in the 1960's. While studying demineralized bone matrix implants and their effect on animal models, researchers discovered that these proteins could somehow induce the formation of bone and cartilage. As research on BMPs progressed, it was determined that the role of these proteins encompasses much more than just bone and cartilage, and are in fact, major coordinators of developmental processes associated with other organs such as the heart, esophagus, and kidneys [1].

Bone Morphogenetic Protein-2 (BMP -2) has garnered much interest in the regenerative medicine field due to its osteoinductive capabilities and is currently used in a therapeutic capacity for the stimulation of bone growth in patients undergoing maxillofacial surgeries, spinal fusion, and tibial fracture fixations [2].

For the purpose of bone regeneration, BMP-2 mediates a specific signaling pathway, in which the BMP -2 binds to a type 2 BMP receptor (BMPrII) present on the membrane of the cell (Figure 1). The BMP-2/BMPrII complex phosphorylates a type 1 BMP receptor (ALK2, ALK3, or ALK 6), initiating its activation. From there, the type 1 BMP receptors phosphorylate Smad proteins present within the cell cytoplasm[1]. There are several types of Smad proteins, though for the BMP-2 pathway, just Smad 1,5 and 8 are involved. Upon phosphorylation, Smad 1, Smad 5, and Smad 8 accumulate in the nucleus, and act as transcription factors, stimulating Transforming Growth Factor-β

(TGF-β) gene transcription, and subsequent production of TGF-β proteins[2]. TGF-β is involved in many physiological processes, including embryogenesis and cell apoptosis. In the BMP-2 pathway, TGF-β upregulates the differentiation of osteoprogenitor and mesenchymal cells into osteoblasts which are responsible for bone formation [2, 3].

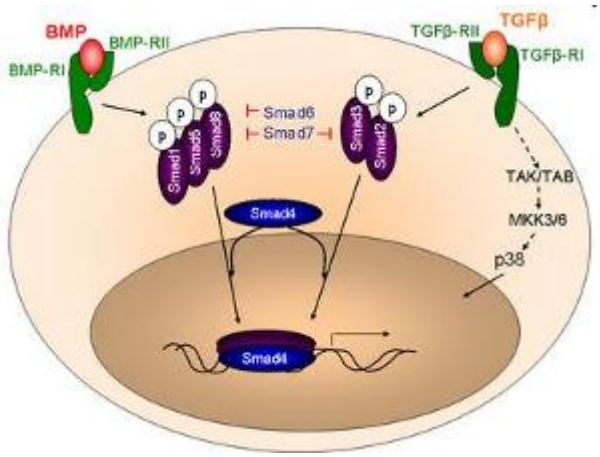

Figure 1: Representation of the BMP-2 Pathway [5]

Long bone fractures comprise nearly 10% of non fatal injuries that occur in the United States per year, where approximately 492,000 of those fractures are associated with the tibia [4]. The most common procedures for treating tibial fractures include immobilization with a fiberglass/plaster cast or intramedullary nailing. The latter is reserved for more serious, open fractures as well as certain fractures that exhibit complex patterns of breakage. The intramedullary nailing procedure involves the insertion of a metallic nail composed of stainless steel or titanium into the intramedullary canal of the

tibial shaft. Following insertion, the nail is stabilized with metallic screws which can be seen in Figure 2.

Figure 2: Postoperative x-ray image of an intramedullary nail inserted into a fractured tibia, stabilized with proximal and distal locking screws [5]

The plaster/fiberglass cast fully supports the bone and renders the patient immobile, but in the case of intramedullary nailing, the metallic nail shares the load with the bone in addition to providing a wider range of leg movement. This early mobilization and application of forces on the healing tibia that are typical of everyday use allows patients treated with intramedullary nailing tend to exhibit shorter recovery times, and less functional disability compared to those treated with casts [6].

In most cases, tibial fractures treated with intramedullary nailing heal within 3 months. Generally, after 5 months of incomplete healing a fracture is considered to be in a state of delayed union, and after 6 months, a non-union. This is of course subject to surgeon discretion on a case by case basis [7]. Delayed unions and non-unions occur due to a variety of reasons and incidences occur in an estimated 10% of tibial shaft fracture cases. Smoking, use of anti-inflammatory medications, bacterial infections, and poor nutritional status are major contributors to instances of delayed or non-unions. The degree of fracture severity can effect healing as well. Compared to closed fractures, open fractures 5 cm or larger in length are 5.7 times more likely to lead to a delayed union or non-unions [8].

Treatment of non-unions often requires costly interventions such as re-fixation, autologous bone grafting, or pharmacotherapeutic delivery of growth factors to stimulate bone formation [4]. Pharmacotherapy involves the administration of chemically or biologically derived drugs, and is regarded as on of the most important methods of treating diseases and injuries. Thus far, over 15 Bone Morphogenetic Proteins with the capability of stimulating bone growth in various types of bony defects and assisting in the healing process have been uncovered, and recently, the FDA has approved BMP-2 for the treatment of open fractures of the tibia stabilized with intramedullary nailing [9]. This, in addition to recent advances in recombinant DNA technology allow for the production of human recombinant BMP-2 (rhBMP-2) at sufficient purity, volume, and reduced cost for therapeutic use, making the controlled delivery of BMP-2 an attractive method of regenerating bone [10].

1.2 Need for Controlled Drug Delivery

Though new drugs for pharmacotherapy garner a great deal of excitement in the medical field, it is equally important that controlled delivery systems are developed to allow for safe, effective, and reliable administration of these drugs into patients.

The therapeutic window of a drug is defined as the range of a drug dosage that will effectively treat the disease or injury, which is illustrated in Figure 3a. Too high of a dosage can increase the chances of adverse side effects, and too low of a dosage will provide less than optimal drug efficacy.

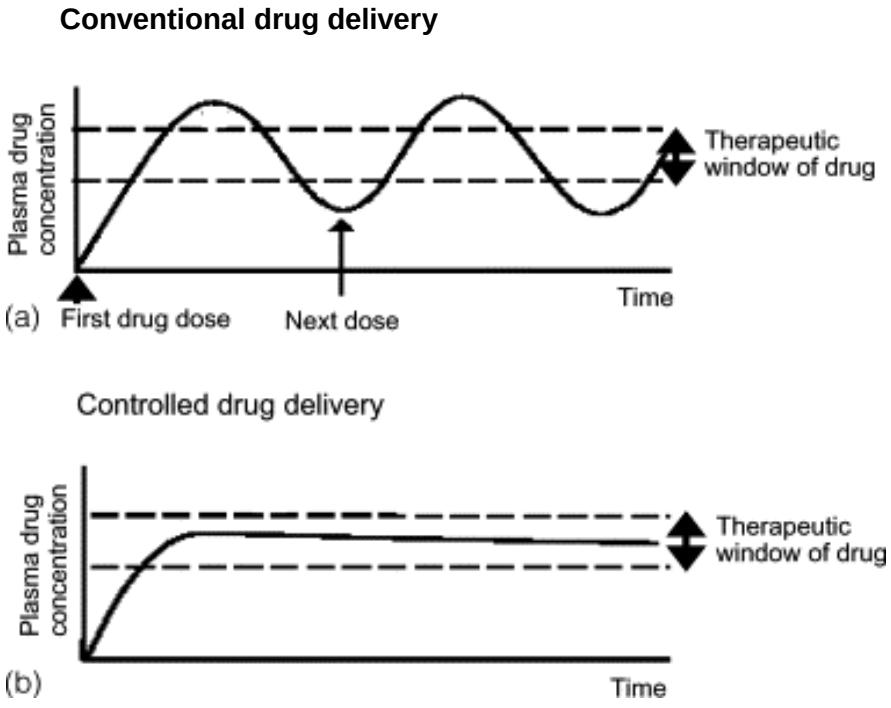

Figure 3: (a) conventional delivery vs (b) controlled delivery with regard to the therapeutic window of the drug [11]

Conventional drug delivery involves oral administration of drug periodically. For example the schizophrenia drug, Haloperidol, is administered orally in 1-5 mg doses every 4 to 8 hours, resulting in fluctuating serum drug levels through the duration of treatment. The peaks and troughs seen in Figure 3 are characteristic of this type of dosing regimen, where serum drug levels oscillate and only fall within desired therapeutic window at certain times after taking the oral dosage form [12].

Controlled delivery technologies are developed to improve drug efficacy (i.e. maximizing therapeutic activity while minimizing adverse side effects). This can be accomplished through a variety of means. For example, some sustained drug delivery technologies provide long release durations to eliminate the requirement of repeated injections, which can improve patient compliance and the oscillations in serum drug levels associated with oral drug intake (figure 3b). Some technologies provide drug release in response to specific environmental stimuli, such as Phenylboronic acid grafted chitosan based drug delivery systems that release insulin in response to free glucose. This allows drug to only be released when it is required [13]. In addition, some drug delivery systems are designed to provide localized release to a specific area of the body, lowering the treatment costs, as well as the overall systemic concentration of drug during treatment as compared to systemic treatment through intravenous (IV) delivery. A prime example of this is technology is the recent development of gentamicin poly-(D,L-lactic acid)-coated intramedullary nails for the surgical fixation of open tibial fractures. This polymeric coating prevents bacterial colonization of the implant and infection while providing nearly undetectable gentamicin serum concentrations [14].

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1.3 Current BMP-2 Delivery Devices for Orthopedic Applications

BMP-2 delivery devices fall into two basic categories:

1. The final product is a device that requires BMP-2 to be incorporated into it at the time of surgery
2. The final product is a device that contains pre-encapsulated BMP-2

1.3.1 Analysis of Category 1 Devices

The only known FDA approved category 1 device is the Medtronic INFUSE Bone Graft. This device is a two component BMP-2 delivery system indicated for use with open tibial fractures stabilized with stainless steel/ titanium tibial nails to induce bone growth. Application of the Bone Graft during the intramedullary nailing operation is used under surgeon discretion as a preemptive method meant to minimize the chances of future surgical interventions due to a delayed union or non-union [15]. The Bone Graft is supplied in the form of a kit containing 2 major components, a vial containing 12 mg of lyophilized rhBMP-2 and a small Type I collagen sponge. The rhBMP-2 is supplied in the lyophilized form for improved shelf life stability. During the tibial nail fixation procedure, the surgeon must reconstitute the lyophilized rhBMP-2 by injecting sterile water into the vial and mixing briefly, creating a 1.5mg/ml solution of reconstituted rhBMP-2. The reconstituted rhBMP-2 is then transferred into a new syringe and the solution is deposited onto a collagen sponge, which quickly absorbs the rhBMP-2 into its porous matrix, which can be seen in Figure 4.

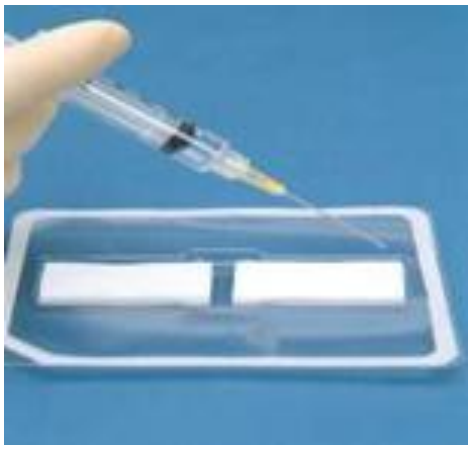


Figure 4: Addition of reconstituted rhBMP-2 solution onto collagen sponge of the INFUSE Bone Graft[16].

The collagen sponge is then applied to the defect site, and the wound is surgically closed. Over the next two weeks, rhBMP-2 will be released into the fracture, inducing its therapeutic benefits [15].

The INFUSE Bone Graft has proven to be efficacious in clinical studies, though some notable issues with the design need to be addressed. For example, the sponge exhibits minimal protein retention due in part to the absence of a method to immobilize the rhBMP-2 within the collagen matrix, such as a crosslinking agent. Additionally, the collagen sponge's release profile is often unpredictable, with high variability observed amongst several devices prepared in the same manner [17]. Furthermore, an inevitable loss of some of the rhBMP-2 upon handling and irrigation associated with the surgical procedure will occur, wasting the expensive therapeutic agent. The absence of a binding agent, allows for a large burst release to occur following the implantation of the loaded sponge, increasing the chances of adverse side effects such as inflammation, massive

swelling, ectopic bone growth, infection, chronic pain, and sterility [17]. In fact, the delivery half life was determined to be very short, occurring within 7-16 minutes upon placement within the body. Therefore, a higher loading dosage (12 mg of rhBMP-2/implant) is required to allow for enough BMP-2 to be released for therapeutic effect during the later periods of the delivery duration [18]. All of these factors contribute to the high cost of the procedure.

The INFUSE Bone Graft does however offer certain beneficial aspects. The incorporation of BMP-2 into the sponge is very straightforward and does not require extensive preparation methods that would compromise sterility. The lyophilized rhBMP-2 and collagen sponge were developed to be prepared individually and packaged in separate containers until time of surgery, which from a manufacturing/industry standpoint is very beneficial, since each component requires different processing and sterilization techniques[18]. Additionally, the INFUSE Bone Graft utilizes a Type I collagen matrix, a natural biomaterial that exhibits hydrophilicity, low immunogenicity, and biodegradability. Type 1 collagen is present normally in significant quantities within cortical bone, so the INFUSE collagen sponge is a logical choice for use as a scaffold material for bone regeneration [19].

1.3.2 Analysis of Category 2 Devices

BMP-2 delivery devices that fall into category 2 are often susceptible to reduced protein stability, partial protein denaturation, and unwanted protein aggregation, due to the extensive processing required to encapsulate the BMP-2 within one or more

polymers, to form a composite polymer - BMP-2 end product. The delicate protein is often exposed to wide temperature and pH changes, as well as harsh, toxic solvents necessary to mold the polymers into the desired conformations. In addition, even if the final product is fabricated without destroying the protein, the shelf life is often shortened since the BMP-2 was likely reconstituted more than once during processing and packaging. Lastly, sterilization of the composite polymer-BMP-2 end product is an issue, since conventional Ethylene Oxide (EO) and Gamma irradiation sterilization techniques used to sterilize polymers are known to degrade BMP-2 [20]. One commendable quality exhibited in nearly all of the category one devices investigated is that in dissolution experiments they exhibit well controlled, predictable release profiles, which is a documented issue with the INFUSE Bone Graft [20-23].

Category 2 devices that rely on natural phenomena rather than specialized processing to incorporate BMP-2 have been developed, which overcome some of these drawbacks. For example, self aggregating specialized lipid microtubules were fabricated by Johnson et. Al [24]. When the microtubules were placed in a BMP-2 solution, uptake of BMP-2 occurred through simple capillary action and subsequent biologically induced degradation of the ends of the microtubules facilitated the release of BMP-2. The BMP-2 loaded microtubules produced a zero order delivery profile, and no burst release was observed. The microtubules performed well in several *in vitro* bioactivity assays as well. This technology gained an edge over other Category 2 devices in that the protein is incorporated into delivery vehicles through a natural phenomenon within an aqueous medium, therefore the BMP-2 was not exposed to any harsh processing procedures. However, capillary action occurs rather slowly, and a 12 hour submersion period of the

tubules was required for them to fully take up enough protein. This would be too long for a device meant for an in situ surgical application [24]. Currently all category 2 devices are experimental in nature and are not in clinical use.

Chapter 2: Design Goals and Specific Aims

2.1 General Design Goal

The deliverable entity of this thesis was a design and prototype for a novel, biodegradable device capable of being loaded with a reconstituted model protein within a 20 minute time span, that exhibits the two component design principle of the INFUSE Bone Graft, but provides controlled release within a more clinical relevant dosage of 1.37 µg to 25.7 µg of protein per day for at least 14 days with no burst release.

2.2 Specific Aims

Specific Aim 1: Develop a novel, dual component drug delivery device comprised of (1) a biodegradable drug reservoir that also acts as a platform for controlled release and (2) a vial containing lyophilized model protein which can be reconstituted and loaded into the drug reservoir. The two components are to be combined at the time of use.

As this is a design project conducted for Synthes Inc, industry requirements must be accounted for. The design must provide for sterilization and a maximal shelf life, therefore the BMP-2 will be stored in its stable, lyophilized form in a separate vial and only reconstituted intraoperatively. Per the requirement of all implantable medical devices, the product will eventually require a specific, validated sterilization method. Accommodations to this requirement are more easily conducted when the two major components are packaged and kept separate until the time of use [21]. Biodegradability

is an advantageous property that many biomaterials exhibit, as it eliminates the need for repeat surgeries to extract the implant after the therapeutic period. Three biocompatible polymers with well established histories of use in FDA approved medical devices were chosen for the device design: polycaprolactone, PEG 400 and sodium alginate. These polymers are eliminated from the body through natural means after implantation. In addition, each material presents its own unique properties integral to the device's performance, which will be discussed in detail in the subsequent section.

Specific Aim 2: Modify the delivery device to be capable of being loaded with a solution of the model protein, bovine serum albumin within a 20 minute time span.

The intramedullary nailing procedure is a time sensitive surgery, and the longer the procedure, the higher the chances that undesirable events could occur, such as bacterial contamination and blood loss. To minimize the time spent in surgery, it is important that the intraoperative preparation procedure for the delivery device be no more than 20 minutes long. This property would also contribute to the novelty of the device, in that no known delivery device can be loaded in less than 20 minutes and still provide release absent an initial burst release [21].

Specific Aim 3: Adapt the device to release 1.37 to 25.7 µg BSA/day for at least 14 days without a burst release.

An optimum pharmacokinetic delivery profile for BMP-2 has not been clinically defined as of yet, though several studies have provided a solid starting point. Wang et. al. found that 2.5 µg of BMP-2 administered per day over 2 weeks was successful in inducing differentiation in D1 and C2C12 cells. They verified their findings by using the same dosage scheme in an *in vivo* experiment involving the healing of tibial mouse fractures [25]. Fu et al. concluded from their study that a sustained release pattern from a BMP-2 loaded scaffold lasting both 2 weeks and 4 weeks provided equivalent bone formation in a nude mouse model upon examination of the mice at week 6 [21]. Research by Hollinger et al. and Johnson et al. also suggest that an initial delivery period without a burst release, followed by a low concentration (~ 2 µg total) controlled release over a minimum of 2 weeks would be a effective in inducing alkaline phosphatase activity in human mesenchymal stem cells. The alkaline phosphatase assay is an established technique often used for assessing in vitro BMP-2 activity, since alkaline phosphatase is one of several early osteogenic differentiation markers [20, 24].

These studies all suggest that a low dose (<1mg over 2-4 weeks) controlled release pattern would be effective for regenerating bone, while minimizing the chances of adverse side effects caused by high dosages. Since a lower loading dosage than that required for the INFUSE product would be implemented, a potentially lower cost would likely be associated with using these devices in a procedure.

It is acknowledged that the optimum therapeutic dosage levels for humans would be different from dosages required for mice or cells in a culture flask, where a few nanograms of BMP-2 released per day can still have a strong effect. Through calculation and educated assumptions made from the combined analysis of the aforementioned

studies, it is estimated that, for an adult patient, controlled release of BMP-2 over at least 2 weeks with ~1.37 µg to ~25.7 µg released per day would be effective in regenerating bone [20, 24, 25]. This particular range is orders of magnitude less than that observed with the INFUSE Bone Graft, where as much as 6,000 µg is released on the first day, followed an excess of 429 µg of BMP-2/day on subsequent days [26].

In general, drug delivery devices that are loaded quickly and not further modified will also release the encapsulated drug quickly. The device for this project was designed to challenge that general trend, having a short loading time while providing a long duration of drug delivery.

Specific Aim 4: Determine the overall material characteristics, in vitro degradation profile, and protein release properties of the device.

As mentioned previously, the desired release profile is an estimation based on published results of *in vitro* and *in vivo* studies. The field of biomedical engineering is constantly changing, and if new information that provides evidence towards a more optimal delivery profile for BMP-2 surfaces, modifications to the release profile may be required. Determining characteristics such as the material degradation rate and the protein release properties from the device will provide a starting point for modifications to the device to increase its therapeutic efficacy. Standardized techniques such as inherent viscosity (IV) testing, gel permeation chromatography (GPC), and scanning electron microscopy (SEM) will be used to observe the material properties device, and the Bradford Coomassie assay for determining protein release.

Specific Aim 5: Verify device compatibility with rhBMP-2.

Due to the high cost of research grade lyophilized rhBMP-2, researchers often utilize lyophilized bovine serum albumin (Fraction V) as a model protein for rhBMP-2 for early testing [27, 28]. Bovine serum albumin is a low cost, readily available protein often employed as a concentration standard for various cellular and protein assays. It is used as a stabilizer for enzymes used in DNA digestion procedures, and is fairly inert from the biochemical standpoint [29]. BSA is 66.5 kDA with 585 amino acid residues, as compared to the smaller 26 kDa BMP-2 protein composed of 114 amino acid residues. A comparison of the tertiary structures of BSA and BMP-2 can be seen below in Figure 5.

BSA BMP-2

Figure 5: Structural Comparison of BSA and BMP-2 Proteins [29]

It is worth noting that the isoelectric point of BSA is pH 4.8 while the isoelectric point of BMP-2 is pH 8.2, therefore the charges of the proteins are opposite at the physiological pH of 7.4 [28]. The differences in overall size, structure, and isoelectric potential between the two proteins were of concern when assessing the appropriateness of using BSA as the model protein for BMP-2 [30]. However, previous studies that utilize

BSA as the model protein show no notable differences between the release profiles of the delivery devices loaded with BSA and those same devices later loaded with BMP-2. In the end, the researchers were successful in using BSA to determine the overall release trends of the device until they were able to test it with BMP-2 [27,28].

During the early testing period of this project, lyophilized BSA was to be used as a model protein for evaluating the loading and release properties of the device. Once several promising formulations were developed that were able to produce the desired release profile with BSA, the device was to be loaded with lyophilized BMP-2 and the release evaluated with a BMP-2 specific ELISA and the alkaline phosphatase assay, to verify that the desired release profile and overall bioactivity of the encapsulated BMP-2 was retained.

2.3 Design Criteria and Constraints

Shown in Table 1 are the criteria and constraints of the design project concluded from development of the specific aims. Included are the details as to why each criterion and constraint was chosen, and whether the success of each criterion/constraint was verified through testing.

Table 1. Summary of Design Project Criteria and Constraints

Criteria	Details	Verification
The device must have an in situ loading procedure similar to the INFUSE Bone Graft, with a preparation time of no longer than 20 minutes	In situ loading DDS in theory will have the benefit of simplified manufacturing, sterilization procedures, and longer shelf life compared to technologies requiring pre-encapsulation of the protein.	✓
The DDS must provide 1.37 - 25.7 µg of protein per day for a minimum of 2 weeks, with no discernable burst release	A theoretical therapeutic release profile was generated based on the analysis of *in vivo* and *in vitro* studies meant to determine the most efficacious delivery profile for BMP-2	✓
Constraints	**Details**	**Verification**
Only readily available, biodegradable/bioresorbable materials with established histories of clinical use may be used in the device design	This device is intended for eventual implantation, therefore only biomaterials with histories of clinical use shall be chosen for device fabrication.	✓
The device design must be novel	Since this is a Synthes funded project, it is important that intellectual property is generated for future benefit to the company. The device will be developed after extensive patent and literature research.	✓

Chapter 3: Design Concept of PCL : PEG 400 Delivery Pouch with Internal Alginate Sponge

Controlled drug delivery involves the precise introduction of an agent into a subject to elicit therapeutic effects. Various types of biodegradable polymers and processing technologies have been utilized in the formulation of delivery devices to promote various properties. Examples includebinding drugs to implantable scaffolds to provide localized delivery to target area, and the formation of specialized coatings to improve drug absorption [31, 32].

Synthetic and natural polymers are used to a great extent in the development of controlled drug delivery technologies. The device design specific to this project combines the properties of polycaprolactone, polyethylene glycol, and sodium alginate.

3.1 Properties and Applications of Polycaprolactone in Drug Delivery

Polycaprolactone (PCL) is a semicrystalline, biodegradable polyester with a well established history of use in the biomedical field [31]. Polycaprolactone has traditionally been synthesized through the ring opening polymerization reactionof ε-caprolactone with the aid of a stannous octoate catalyst [32]. The five $–CH_2$ groups in each repeating unit impart a net hydrophobicity to the PCL (Figure 6).

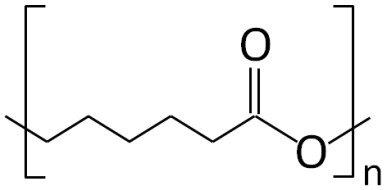


Figure 6: Structure of the polycaprolactone repeating unit [32]

In addition, the long, tightly packed chains that form from the linked monomers provide a high degree of mechanical strength. The tensile strength of PCL can range from 10.5 – 16.1 MPa with the modulus ranging from 343.9 – 364.3 MPa, making it an appropriate choice for specialized load bearing applications and applications requiring material durability [33]. In vivo degradation of PCL occurs due to hydrolysis of the ester linkages to form 6-hydroxycaproic acid, which is then converted to adipic acid by the liver and kidneys, followed by metabolization of the adipic acid via the citric acid cycle to form water and carbon dioxide [34]. Overall, the polymer exhibits low toxicity in many clinical studies as well as a slow degradation time (~2 years) [32].

PCL has several characteristics that aid in its processability. The polymer is a tough plastic at room temperature, but it can be melt cast and shaped to a wide variety of specialized conformations at temperatures greater than 60˚C due to its low melting point (58-60 ^{o}C) [35]. PCL can be dissolved in several common organic solvents such as dichloromethane and chloroform at room temperature as well, further expanding its applications as a viable biomaterial.

For these reasons, PCL is often used to create a wide variety of biomaterial technologies such as electronspun scaffolds, biodegradable sutures, and the hydrophobic

portions of polymersomes[36]. Several PCL based drug delivery systems are comprised of PCL blended with other natural and synthetic materials to create novel composites exhibiting combinations of desirable properties. PCL has been combined with chitosan to impart higher hydrophilicity and faster degradation (i.e. faster drug release), acrylic acid for environmental sensitivity to pH, and hydroxyapatite to improve tensile modulus of the drug delivery system. [31, 37, 38]

3.2 Properties and Applications of Sodium Alginate in Drug Delivery

Sodium alginate is a naturally derived, anionic polysaccharide extracted from the cell walls of brown algae. Chemically, sodium alginate is characterized as a linear polymer composed of β -D-mannuronic acid and α -L-guluronic acid monomers which is depicted in Figure 7. The arrangement of the monomers is limited to homopolymeric G blocks, homopolymeric M blocks, and alternating, heteropolymeric MG blocks [39].

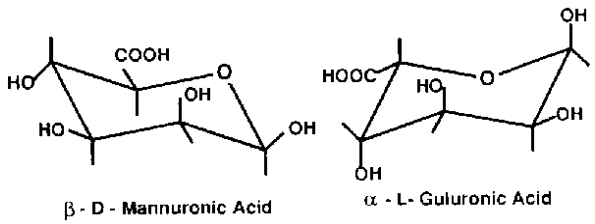


Figure 7: Structure of beta-D-mannuronic acid and alpha-L-guluronic acid monomers [29]

Sodium alginate in an aqueous solution forms a clear, viscous liquid at low concentrations (<2% w/v in distilled water), and increasing concentrations of alginate corresponds with higher viscosity solutions. A solution of sodium alginate can be crosslinked when exposed divalent ions, such as ionic calcium (Ca^{2+}). The calcium ions will replace the sodium present in repeating units of guluronic acid in adjacent polymer chains, inducing the formation of a 3D hydrogel network described as the egg box model (Figure 8).

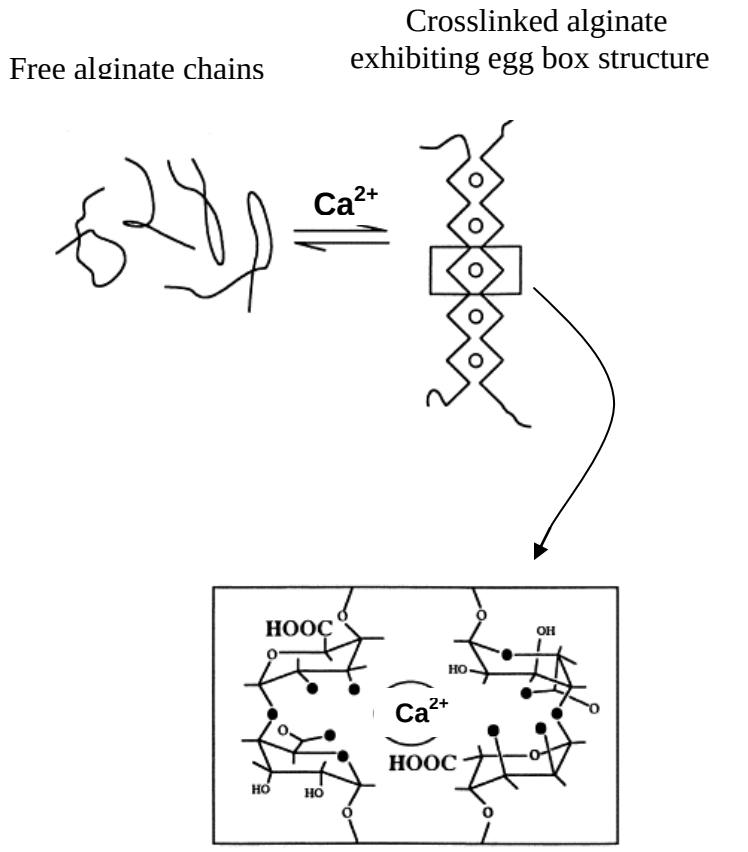


Figure 8: Representation of egg box model formed with sodium alginate and Ca^{2+}[40]

Alginate hydrogels are highly hydrophilic, capable of swelling and retaining over 200 times their weight in water, allowing them to be useful in a wide array of applications [41]. The physical properties of sodium alginate are dependent on the ratios of G and M blocks. For example, since G blocks are involved in calcium ion induced crosslinking and gelation, a higher proportion of G blocks results in higher gel strength and rigidity. The elasticity of the alginate corresponds to the proportion of M blocks, in that the higher the proportion of M blocks, the more elastic the gel [39].

Sodium alginate has exhibited low immunogenicity and good biocompatibility making it a viable choice as a biomaterial. Sodium alginate has been utilized in a variety of biomedical engineering applications such as a component in scaffolds for tissue engineering, where they are blended with other polymers such as chitosan, poly(lactic-co-glycolic acid) (PLGA),and PCL to promote cell adherence [42]. Sodium alginate is often used as a controlled release material for low molecular weight drugs, where the drug is encapsulated within the alginate gel structure, and diffusion facilitates release of the drug out of the alginate structure. Sodium alginate is also suitable for technologies involving protein encapsulation, due to the stable, hydrophilic internal environment provided by the gels [43]. Furthermore, release profile can be modulated with the addition of other degradable polymers or processing (e.g. pre-oxidation of alginate to facilitate quicker drug release). Crosslinking agents for sodium alginate are not limited to calcium ions alone, and crosslinking can readily occur with a variety of non-toxic solutions containing divalent cations. It is for this reason that alginate is often chosen for the encapsulation of

delicate proteins since many other hydrogel systems require harsh, toxic crosslinking agents that induce protein denaturation [44].

3.3 Properties Applications of Polyethylene Glycol (PEG) in drug delivery

Polyethylene glycol (PEG) is a non-toxic, hydrophilic polymer employed in a wide spectrum of biomaterials and therapeutic agents. For example, it is used as an emulsifying agent in skin creams, a coagulant in wound dressings, and the stealth component in liposomes for drug delivery. Depending on the molecular weight, PEG can be clear, viscous liquid (e.g. PEG 400) or a white solid (e.g. PEG 3350) at room temperature [45].

PEG is synthesized through the reaction of ethylene oxide with water or ethylene glycol oligomers, in the presence of an acidic/basic catalyst, forming a polymer with repeating ethylene oxide units arranged in linear or branched chains as shown (Figure 9) [46].

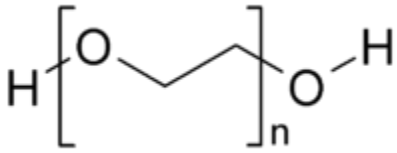


Figure 9: Structure of Polyethylene Glycol repeating unit [46]

After injection or implantation within the body, PEG exhibits high biocompatibility and is not known to elicit systemic toxicity, though due to its chemical structure, PEG is non-biodegradable *in vivo*. However, PEG 400 can be cleared from the body quite easily through glomerular filtration and biliary excretion and is therefore an FDA approved additive in many drug and food preparations. In drug delivery, PEG is often conjugated to proteins and non-peptide molecules in a process known as PEGylation to increase the *in vivo* half life, allowing for the modification and potential improvement of the pharmacokinetics/ pharmacodynamics of an administered drug [47]. PEG can be blended with a wide variety of polymeric materials due to its solubility in water and most organic solvents, making it a logical choice for the fabrication of many composite materials [48].

3.4 General Design Concept

The general design concept of the device consisted of a two major parts: (1) an outer polymeric pouch composed of a blend of a slowly degrading, mechanically strong polymer and a water miscible polymer that acts as a porogen, and (2) a protein absorbing sponge insert composed of a hydrogel material (Figure 10).

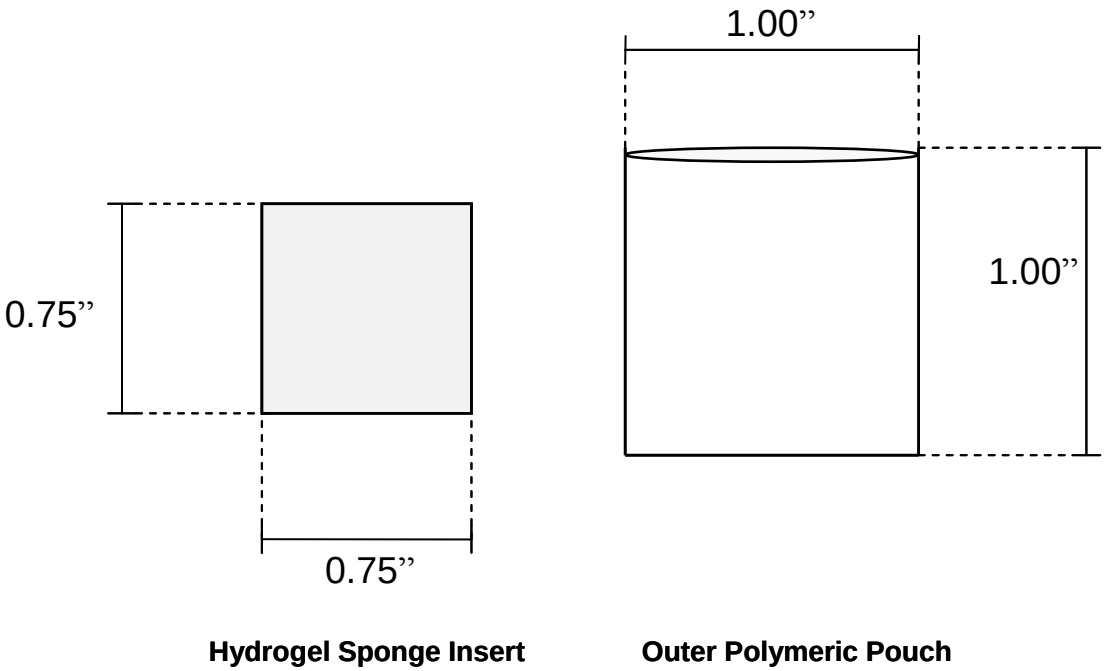


Figure 10: Early design schematic of outer polymeric pouch and hydrogel sponge insert

The initial design concept was then translated into an engineering drawing, which can be seen below in Figure 11.

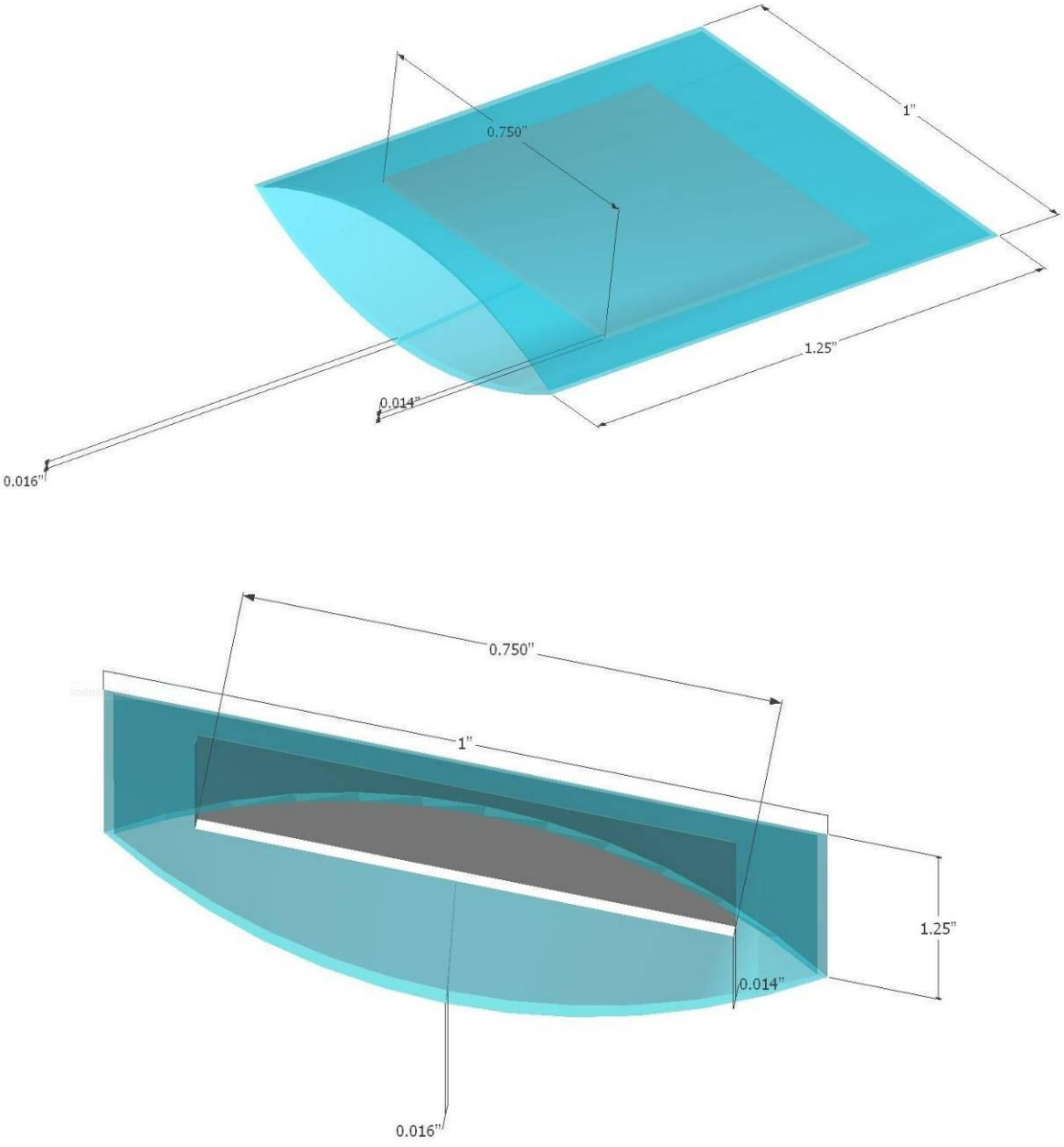


Figure 11: Engineering Drawing depicting the polymeric pouch and the inserted hydrogel sponge. The side view and front view of the pouch are shown.

The general preparation method for the device during a tibial nailing procedure can be seen in Figure 12 A-D. First, the surgeon would insert the sponge into the pouch, followed by reconstitution of the supplied vial of BMP-2 with sterile water (A). The surgeon would then inject the solution into the open end of the pouch via syringe, where the sponge will quickly absorb the BMP-2 solution (B). The surgeon would then inject crosslinking agent into the pouch to crosslink the sponge (C). Lastly, the pouch will be sealed tightly, where it will be ready for application to the tibial fracture (D).

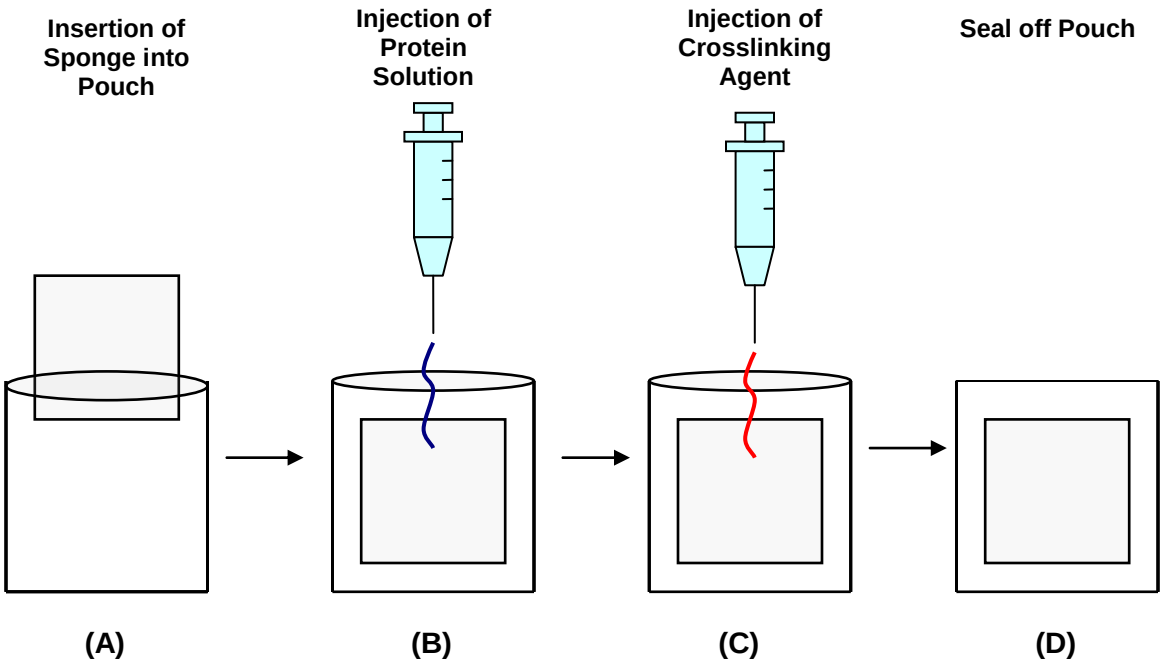


Figure 12: Basic preparation scheme for delivery pouch

After implantation, the water miscible portion (ie. the porogen) of the pouch would dissolve out, leaving behind a network of small pores within the slowly degrading

polymer. The porous polymer material would then act as a rate controlling membrane, influencing the rate in which the encapsulated protein could diffuse out of the hydrogel and into the adjacent fracture space (Figure 13). Theoretically, the release rate would be modifiable by altering the ratio of the slowly degrading polymer and the water miscible polymer that are used to create the pouch.

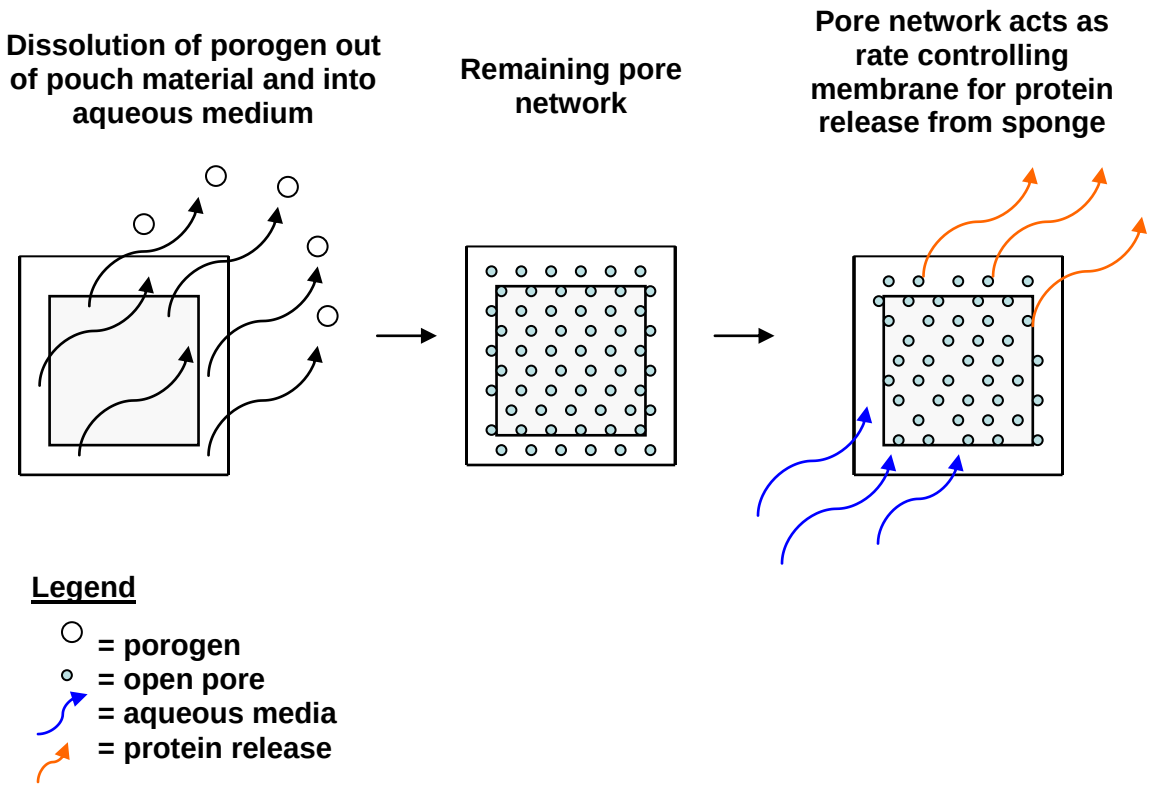


Figure 13: Illustration of controlled protein release from pouch after formation of pore network.

The following section briefly summarizes the general method development and scientific theories used to create functional prototypes of the pouch and sponge prior to

the testing. For the comprehensive fabrication methods used to create the specific pouches that participated in the actual testing, see section 4.1.

3.4.1 Pouch Material

It was determined that the pouch portion of the device was to be composed of thin films created from a blend of polycaprolactone (slowly degrading polymer) and PEG 400 (water miscible polymer). This would be accomplished by first dissolving the solid polycaprolactone in chloroform at room temperature, followed by the addition of PEG 400 into the mixing vessel. It was expected that a homogeneous solution of polycaprolactone and PEG 400 would be produced due to the miscibility of PEG 400 in chloroform. To start, a 90:10 volume ratio of PCL to PEG 400 was planned for testing, with the intent that later studies would be done to optimize the PCL : PEG 400 ratio.

The PCL: PEG solution would then be poured into a rectangular polypropylene mold, enough to cover the bottom surface of the mold (liquid depth ~2mm). The entire mold would then be placed in a solvent oven at room temperature to allow the chloroform to evaporate, leaving behind a solid rectangular film composed of PCL interspersed with PEG 400.

As mentioned previously, PCL has a low melting point of 58-60ºC. To make the pouch, it was theorized that the films could be heat sealed together using a heat sealer set to 70˚C, which was above the melting point of PCL. Two square pieces approximately 1.25” x 1.25” in dimension would be cut from the master film and placed back to back.

Then three of the four sides would then be sealed, leaving one opening for injection of the protein solution.

This proposed formulation would be interesting in that a slowly degrading, high molecular weight, hydrophobic polymer would be homogeneously blended with a low molecular weight, hydrophilic polymer to create a composite material embodying the characteristics of both materials. Since the PEG 400 is soluble in aqueous solutions and the polycaprolactone is insoluble, it was surmised that once the pouch was placed in aqueous media (e.g. PBS) the PEG 400 would immediately dissolve out, leaving behind the PCL with a distributed pore network. The porous PCL material would then act as a rate controlling membrane for protein diffusion out of the sponge and into the surrounding solution. It was expected that the release rate would increase with higher ratios of PEG 400 to PCL. One of the main reasons as to why PEG 400 was chosen was due to its miscibility in both chloroform and aqueous solutions. Miscibility in chloroform would allow for extremely even blending of the materials during fabrication and eliminate the need for any emulsifying agents.

3.4.2 Alginate Sponge

Preliminary prototyping was conducted to develop the device's sponge component and it was discovered that a simple procedure could be used to create the sponge, which involved first dissolving powdered sodium alginate in DI water (1% w/v). The solution was poured into a square plastic tray, then frozen and subsequently placed under vacuum to cause sublimation of the trapped water to produce a white, fibrous mat

of alginate that exhibited the same shape as the plastic tray. This mat had the consistency of a cotton pad and could easily cut into a variety of shapes.

From literature research, a 1:2 w/v ratio of sodium alginate to $CaCl_2$ was able to successfully crosslink alginates in solution [49]. To ensure rapid crosslinking of the sponge insert, therefore a faster intraoperative preparation time, a higher concentration of crosslinking agent than that reported in literature (10% w/v) was chosen for initial testing purposes, with the intent that this be optimized later in future experiments.

3.4.3 Intraoperative Sealing Method

The pouch would come to the surgeon with three sides already sealed and the fourth unsealed, with the sponge simply sitting inside the pouch itself. A surgeon would not have a heat sealer present in the operating room, therefore a method was required for sealing the fourth side intraoperatively after the BMP-2 and crosslinking solutions were injected into the pouch. This seal would need to durable and water tight, to ensure that the release rate of the device was dependent solely on the pouch material's properties as a rate controlling membrane. Extensive literature research was conducted on medical adhesives and glues but none that were found were appropriate for this particular application due to excessively long curing times or toxicity. This was an important consideration since the delivery device is meant to be implanted within the human body. It was determined that a solvent bonding technique would be a satisfactory approach, as long as the solvent was of extremely low or non-toxicity.

Ethyl acetate ($CH_3COOCH_2CH_3$) is a colorless organic solvent manufactured through the esterification of ethanol and acetic acid. It is present in significant quantities

in fruits, wines, and certain glues, and is often used in labs for biological extractions and chromatography [50]. Both PCL and PEG 400 are soluble in ethyl acetate, and ethyl acetate exhibits high volatility. It was determined that applying a small amount (<20 µl) of the solvent to the unsealed faces of a prototype pouch via a metal spatula, and simply pressing the two sides of the pouch together with thumb and forefinger, allows successful solvent bonding to occur and form a water tight seal. Additionally, the volatile nature of ethyl acetate would allow for the ethyl acetate to simply evaporate at room temperature after fulfilling its sealing function [50]. In the surgical setting, the surgeon would likely be supplied with a small glass vial of ethyl acetate with a brush applicator tip built into the cap.

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) integrates the regulatory bodies of Europe, Japan, and the United States to synchronize and clearly define the requirements for medical product development and registration. ICH guidelines are accepted as law in numerous countries and are considered key standards by the FDA [51]. According to ICH, ethyl acetate is a class 3 solvent, that is, a solvent of low toxic potential and minimal health risk. Under the guidelines, exposure to residual ethyl acetate up to 50 mg per day is acceptable [51]. The amount of ethyl acetate used for sealing the pouch is far below this level, being 20 µl at most (i.e. 14.4 mg). After sealing the pouch, the ethyl acetate is expected to evaporate, which will decrease the residual solvent level in the implant to an even lower value.

Chapter 4: Preparation of PCL : PEG 400 Pouch and Alginate Sponge Insert

4.1 Fabrication of PCL: PEG 400 composite pouch

Note: For all experiments and protocols discussed in this and subsequent chapters, model numbers and suppliers associated with each piece of equipment can be found in Appendix A-D1. For materials, the product names, suppliers, and lot numbers can be found in Appendix A-D2.

Polycaprolactone granules (65,000 avg MW) were dissolved in chloroform at the ratio required to create a 10% w/v solution. 9 ml of the 10% PCL solution was mixed with 1 ml of PEG 400 in a glass vial and gently vortexed to create a homogeneous solution of PCL:PEG 400. The mixture was poured into a rectangular 5" x 3"x 1" polypropylene mold, and the entire mold was then transferred to a solvent oven set to room temperature (23˚C). The mold was left overnight (~12 hours) to give more than adequate time for the chloroform to fully evaporate. A flat PCL film with interspersed PEG 400 remained after the chloroform evaporation (Figure 14). The approximate thickness of the film was 0.2 mm thick and measured by a calibrated micrometer.

Figure 14: 90 PCL: 10 PEG 400 composite film after solvent evaporation

Two 1.25” x 1.25” squares were cut from the film and placed back to back in the sealing platen of a PACKWORLD custom heat sealer (Figure 15). The sealing temperature was set to 70° C, above the melting temperature of PCL (58-60^{o}C).

Figure 15: Heat sealing step for pouch fabrication

Three sides of the films were sealed, creating a pouch with one open end (Figure 16). The final dimensions of the pouch was 1” x 1.25” x 0.016”, measured by calibrated calipers.

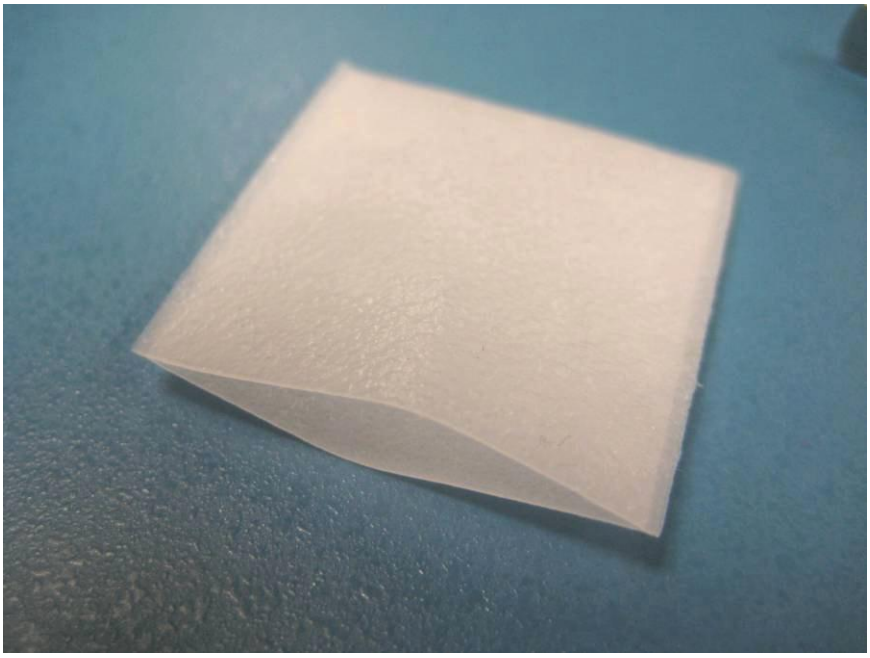


Figure 16: Completed 90 PCL: PEG 400 Pouch

4.2 Fabrication of the Sodium alginate sponge

Sodium alginate powder was mixed with deionized (DI) water to prepare a 1% w/v solution. 45 ml of the solution was poured into a 5" x 5"polystyrene tray and the tray was chilled at -80° C for 5 hours. The tray was then transferred to a vacuum oven at 7 torr and left for 12 hours to extract the water. This freeze-drying process produced a dry, fibrous mat of sodium alginate with a sponge-like consistency (Figure 17).

Figure 17: 0.75” x 0.75” x 0.014” alginate sponge.

As a last step, the 0.75” x 0.75” square was cut from the master sheet and inserted into the PCL:PEG 400 pouch (Figure 18).

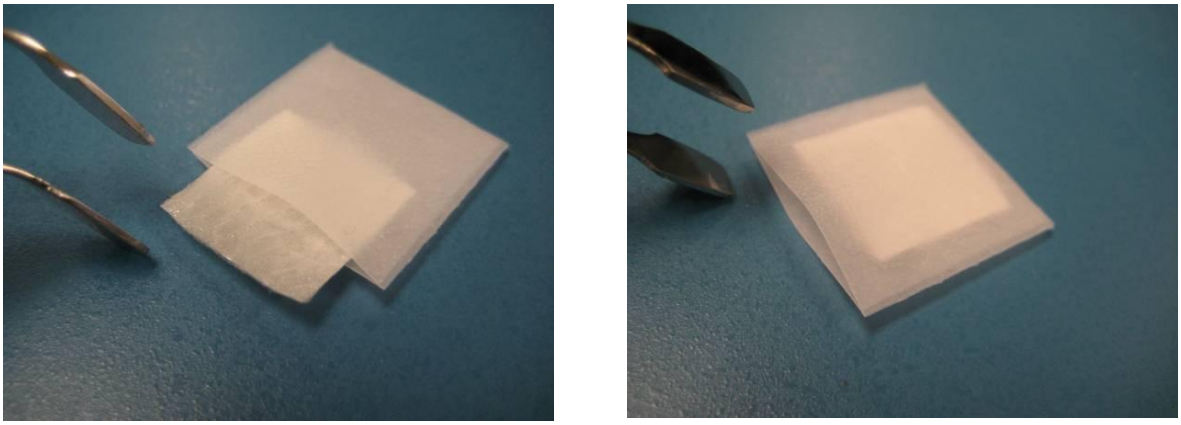


Figure 18: Sodium alginate sponge inserted into PCL: PEG 400 pouch

4.3 Basic Pouch Loading Procedure:

The pouches were loaded with solutions containing varying amounts of reconstituted BSA depending on the experiment. However the procedure in which they were loaded were generally the same. This consisted of weighing out the required amount of lyophilized BSA into a glass vial in the fume hood and adding the corresponding amount of deionized water with a sterile pipette tip. The vial was capped and gently inverted several times to dissolve the BSA. To prepare the crosslinking agent, 1 g of calcium chloride powder was poured into a glass vial along with 10 ml of deionized water (10% w/v). The vial was capped and placed on the vortexer at the highest setting for 10 seconds until the solution became visually homogeneous.

To load the delivery system, 100 µl of the BSA solution was drawn up using a pipette with a sterile tip and injected into the pouch through the pouch's open end (i.e. the mouth

of the pouch). After a 5 minute waiting period, 200 µl of the $CaCl_2$ solution was injected into pouch using the same pipette with a new sterile tip (Figure 19A).

A clean metal spatula was dipped into a glass vial containing 1 ml of ethyl acetate and placed in between the two sides of the open mouth. The two sides were pinched together and the spatula was removed, facilitating a tight solvent bonded seal (Figure 19B). The device was then ready for dissolution testing (Figure 20).

(A) (B)

Figure 19: (A) Injection of 10% $CaCl_2$ solution into completed PCL: PEG 400 pouch with internal sodium alginate sponge, followed by (B) sealing step with ethyl acetate and metal spatula

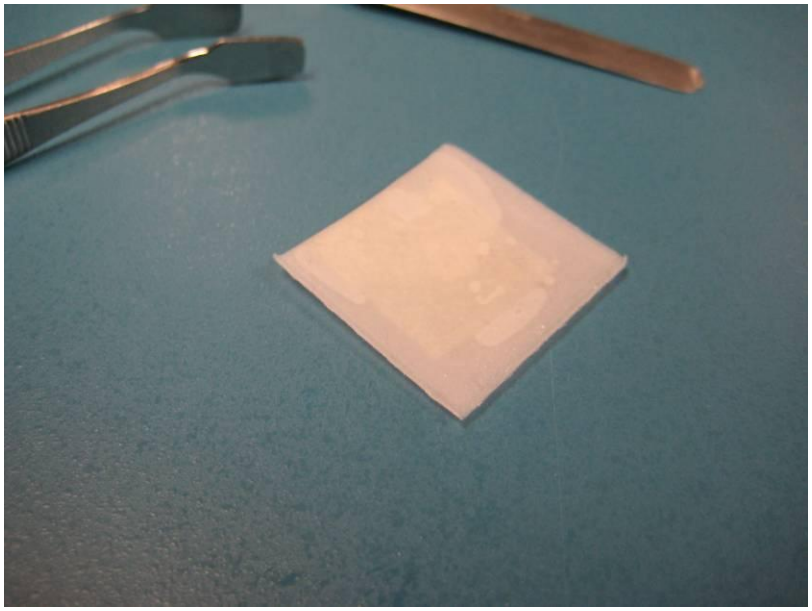


Figure 20: Sealed PCL: PEG 400 Pouch with loaded and crosslinked sodium alginate sponge.

Chapter 5: Method for Evaluating Release Characteristics of BSA from PCL:PEG Pouches

5.1 Background: Coomassie-Bradford Assay for Protein Determination

The Coomassie-Bradford assay is a rapid, established method of quantifying protein in solution. Coomassie Brilliant Blue G-250 is a triphenylmethane based dye that was originally developed for the textile industry [52]. Due to its chemical structure, the dye stoichiometrically binds to the amino acids of proteins, specifically, arginine (R), phenylalanine (F), tryptophan (W), and proline (P), resulting in a color shift (Figure 21)

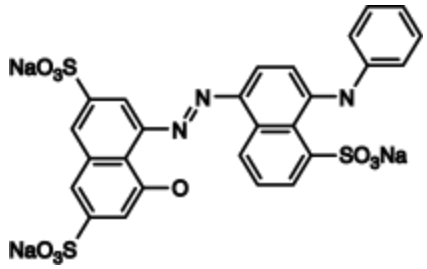


Figure 21: Chemical structure of Coomassie Brilliant Blue G-250 [53]

From a macroscopic visual point of view, the unbound dye is normally dark brown, but becomes blue when bound to protein [54]. The intensity of the shift from brown to blue is dependent on the amount of bound protein in solution (e.g. the more protein, the more intense the blue coloration appears). Unbound coomassie dye exhibits a maximum absorbance at 470 nm, while the bound dye is detected the most strongly at 595 nm [53].

For this particular application, a series of stepwise dilutions from 0 – 25 µg/ml of BSA calibration standard, followed by addition of Coomassie blue to each standard dilution, could be conducted, followed by absorbance measurements at 595 nm. By determining the absorbance readings of each standard and comparing the values to the known concentrations of the linearity standards, a standard curve can be generated. From there the unknown protein concentrations of a set of test samples can be determined based on their absorbance readings [55].

5.2 Standard Method for determining protein release characteristics of delivery pouch

Dissolution testing is commonly used in the pharmaceutical industry to predict the drug release profile of a drug delivery device when placed in conditions that model the internal physiological environment of a human. This is usually accomplished through the use of container filled with a physiologically relevant medium, such as PBS or cell culture medium which is maintained at 37ºC, the average body temperature of a human. Often but not always, agitation is applied to the medium as well. Dissolution parameters are highly dependent on the application, and variables such as medium, pH, and agitation level will vary between researchers [56].

In this project, dissolution testing was conducted to assess the release characteristics of the drug delivery pouch before and after various formulation changes. The same basic dissolution testing scheme was utilized through the duration of this project to maintain experimental consistency and is discussed hence forth. Studies that required dissolution testing disclosed in this document will refer back to this section.

To facilitate protein release under physiological conditions, each new pouch was loaded with the required concentration of BSA solution, crosslinked with $CaCl_2$, and sealed in a fume hood, prior to submersion in a glass vial containing 10 ml of prewarmed phosphate buffered saline (37 °C, pH of 7.4). The vials were then placed in a shaking water bath set to 25 RPM and 37 °C. The medium within the sample vials was replaced every 48 hours with fresh PBS, and the old medium from each of the vials, containing the released protein, was stored, tightly capped, at 4 °C until ready for testing. To begin the testing procedure, the medium within the vials was transferred to 15 ml conical tubes and centrifuged for 5 minutes at 2000 RPM to separate any suspended solids. The supernatants for each of the samples which contain the protein were added to polystyrene cuvettes and mixed with bradford reagent at a 1:1 v/v ratio. The samples were then incubated at room temperature (~ 23 °C) for ten minutes to allow the reaction to occur. During the two waiting periods, seven linearity standards for the experiment were prepared in the range of 0 – 25 µg/ml, as well as a blank cuvette containing DI water (Figure 22).

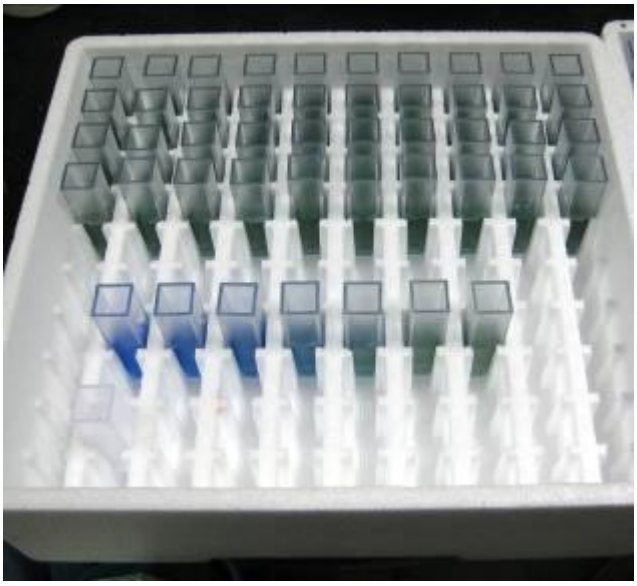


Figure 22: Typical arrangement of dissolution samples and linearity standards containing Bradford reagent before spectrophotometric analysis.

Using a Cary WinVu UV-Vis Spectrophotometer, the absorbances of the samples, standards and blanks were read at 595 nm. A standard curve was made by plotting the absorbance of each BSA standard versus the known concentration in µg/ml. Using the equation generated by the linear trend line of the standard curve, the protein concentration of each sample was determined to generate a dissolution profile for the delivery pouches. An example of a standard curve calculated from the BSA standards can be seen in figure A-C1 in Appendix C.

Chapter 6: Effect of Varying PCL :PEG 400 ratio on Release Characteristics of Delivery Pouch

6.1 Background

Since there is no standardized dosage scheme for BMP-2, the desired release profile specified in the design aims was developed based on current data published in literature. A flexible device design is essential to easily adapt to new information that may arise. In this study, the effect of altering the ratio of PEG 400 to PCL on the release kinetics of the overall device was assessed, to determine whether this was a possible method for modifying the pouch's release profile in a predictable manner.

6.2 Methods

All parameters were kept constant with the exception being the ratio of PCL to PEG 400 used to fabricate the outer film portion of the pouch, defined below. There were three pouches (n=3) contained in each group, and the exact ratios of PCL to PEG 400 used for the experiment can be seen below in Table 2.

Table 2: Ratio of PCL to PEG 400 and loading volume of BSA for each test group

	Pouch Components	
Group Designation	Volume of 10% PCL solution used for pouch fabrication (ml)	Volume of PEG 400 used for pouch fabrication (ml)
90 PCL : 10 PEG 400	9.00	1.00
92.5 PCL : 7.5 PEG 400	9.25	0.75
95 PCL : 5 PEG 400	9.50	0.50
97.5 PCL : 2.5 PEG 400	9.75	0.25
100 PCL : 0 PEG 400	10.00	0.00

.

The same basic fabrication method outlined in Sections 4.1 was followed. All of the pouches in the study were loaded with 100 µl each of a stock 3.00 mg/ml BSA solution, and crosslinked with 10% w/v calcium chloride solution.

The pouches were sealed with ethyl acetate and placed in dissolution vials containing 10 ml of PBS (pH 7.4) and incubated at 37ºC and 25 RPM. 100% medium changes were conducted approximately every 48 hours, and the old medium containing the released BSA was stored at 4 ºC until ready for testing. The dissolution experiment was ended after 16.08 days, and the stored samples were analyzed with the Coomassie-Bradford method.

6.3 Results & Discussion

The average cumulative release of BSA (µg) for each formulation was determined and plotted against the 16.08 days in which the dissolution test took place, and can be seen in Figure 23.

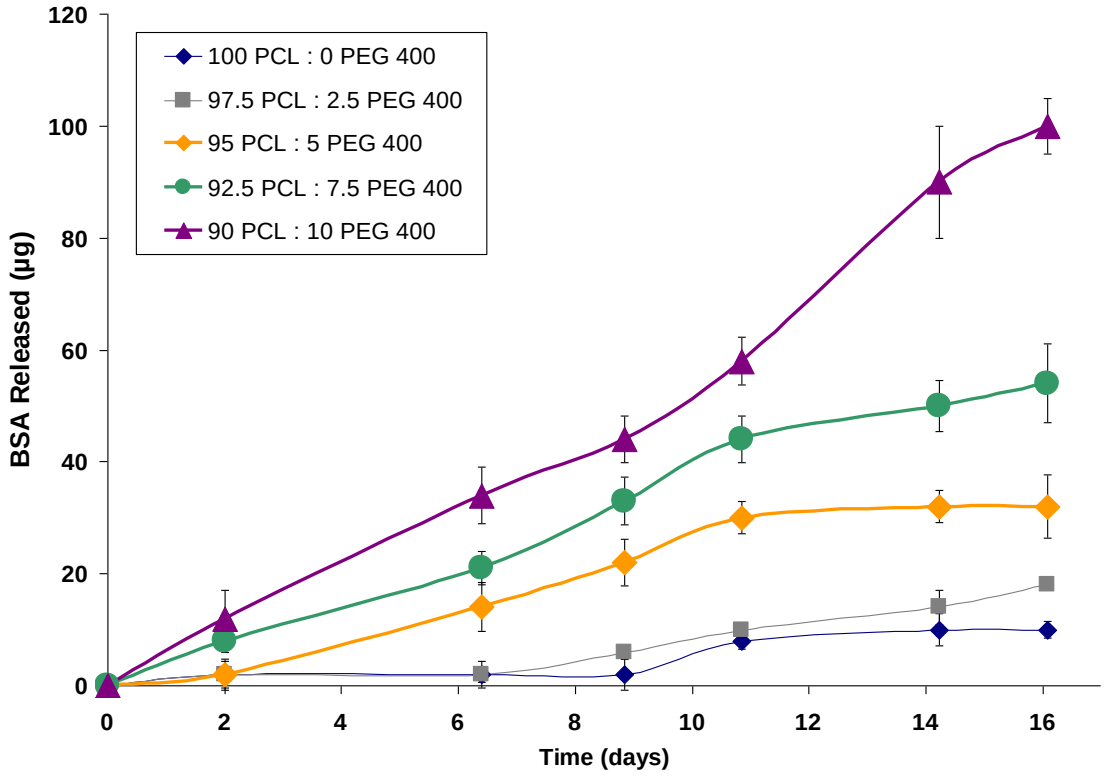


Figure 23: Cumulative Release of BSA in µg from pouches with varying PCL: PEG 400 ratios (n = 3)

Table 3: Average BSA released per day from each group of pouches

Group Designation	Estimated BSA released per day (µg)
90 PCL : 10 PEG 400	5.54
92.5 PCL : 7.5 PEG 400	3.31
95 PCL : 5 PEG 400	2.56
97.5 PCL : 2.5 PEG 400	1.38 (total after day 6.5)
100 PCL : 0 PEG 400	1.25 (total after day 8.8)

The PCL only group (100 PCL : 0 PEG 400) on average, released little to no BSA over the first 8.85 days. This was followed by small amounts of release totaling ~10 µg up to the 16.08 day timepoint. This result was expected due to the formulation's lack of PEG 400. Without PEG 400 present to dissolve out of the PCL and leave open pores, immediate PBS influx into the pouch could not occur to induce an immediate release of BSA. The majority of BSA released in this formulation occurred only after 8.85 days, which can be attributed to the bulk erosion of the PCL itself, which as seen in the results, is a slow process that will eventually produce porosity as well.

Overall there was no statistically significant difference (ANOVA, $p>0.05$) between the release characteristics of the pouches within the 100 PCL : 0 PEG 400 group versus the pouches of the 97.5 PCL : 2.5 PEG 400 group. This phenomenon suggested that the small amount of PEG 400 present in the pouch was not sufficient enough to facilitate open porosity to the degree that substantially more BSA would be released, compared to when no PEG 400 is present. However, when the PEG 400 content was increased to a higher degree, as seen in the 95 PCL : 5 PEG 400 group, the release characteristics changed dramatically, and immediate release of BSA was exhibited. This trend continued with the 97.5 PCL : 2.5 PEG 400 and 100 PCL : 0 PEG 400. The release curves for the four groups showed a significant difference (ANOVA, $P<0.05$). These data demonstrated that for the pouch, the higher the PEG 400 ratio, the higher the rate at which BSA was released.

This study showed how modifying the ratio of PCL to PEG 400 when formulating the PCL : PEG 400 pouch can predictably alter the release characteristics of the completed device. In addition, the 95 PCL : 5 PEG 400, 92.5 PCL : 7.5 PEG 400, and

90 PCL : 10 PEG 400 formulations all deliver BSA in the desired range of 1.37 to 25.7 µg BSA/day for at least 14 days with no burst release, fulfilling Specific Aim 3. In addition, these three formulations were easily loaded with BSA, crosslinked, and sealed within 20 minutes, thereby fulfilling Specific Aim 2.

Chapter 7: Effect of Modified Crosslinking Procedures on Release Characteristics of Delivery Pouch

7.1 Background: Crosslinking methods for sodium alginate

The normal crosslinking procedure for the 1% alginate sponge portion of the delivery device consists of dripping 200 µl of 10% calcium chloride ($CaCl_2$) into the sponge following the addition of the reconstituted protein solution. This method has provided satisfactory effects towards the overall release characteristics of the delivery device.

Before settling on this particular method as the method of choice for all future testing of the device, two additional methods of crosslinking were assessed.

First, rather than adding the reconstituted protein solution to the alginate sponge first, waiting several minutes, then adding the calcium chloride to crosslink, a different approach was attempted. In this endeavor, the 10% calcium chloride solution was mixed with the 3 mg/ml BSA solution to create a single solution of protein and crosslinking agent, so that when the solution was added to the alginate sponge, the incorporation of protein and crosslinking reaction would occur simultaneously. If successful, this would simplify the in situ preparation procedure and minimize the preparation time as well, since only one injection is required.

Sodium alginate based drug delivery devices commonly utilize calcium chloride as a crosslinking agent. However, calcium sulfate is often used as an alternative crosslinking agent to calcium chloride. Both calcium sulfate and calcium chloride act as donators of calcium ions to sodium alginate, however, calcium sulfate is less water soluble than calcium chloride. For this reason, the crosslinking reaction with alginate

utilizing calcium sulfate occurs more slowly, but as a tradeoff, it generally creates more homogeneous hydrogel-drug networks compared to calcium chloride, as well as a slower release rate [57]. It was theorized that a more uniform network would be beneficial in providing a more consistent release profile for the delivery pouch, with less variability. In this study, a high concentration calcium sulfate solution (10%) was used as a crosslinking agent for the alginate portion of the delivery pouch and its effects on the release profile were assessed. The maximum amount of gelation time was allotted (15 minutes) for the crosslinking procedure to occur.

The two alternative methods for crosslinking were compared to the release profile generated from the usual formulation, consisting of the 90 PCL : 10 PEG 400 pouch with the internal 1% alginate sponge that is crosslinked with 10% $CaCl_2$. In addition, the release characteristics of the solitary 1% alginate sponge crosslinked with 10 % calcium chloride, without the outer 90 PCL : 10 PEG 400 pouch was included in the test, for comparative purposes as well.

7.2 Methods

Nine 90 PCL: 10 PEG pouches and twelve 1% alginate sponges were fabricated using the standard method outlined in section 4.1 and 4.2. To prepare the crosslinking agents, 1 g of calcium chloride powder was poured into a glass vial along with 10 ml of deionized water (10% w/v). The vial was capped and placed on the vortexer at the highest setting for 10 seconds until the solution became visually homogeneous. This same procedure was repeated for calcium sulfate. The pouches, sponges, and crosslinking agents were then separated into groups and prepared according to Table 4.

Table 4: Detailed preparation parameters for testing different alginate sponge crosslinking methods.

Group	Number of samples	Pouch material	Sponge material	Crosslinking agent	Loading volume of BSA (µg)
A	n=3	none	1% alginate sponge	10% calcium sulfate	300
B	n=3	90 PCL :10 PEG 400	1% alginate sponge	10% calcium sulfate	300
C	n=3	90 PCL :10 PEG 400	1% alginate sponge	10% calcium chloride	300 (premixed with $CaCl_2$)
D	n=3	90 PCL :10 PEG 400	1% alginate sponge	10% calcium chloride	300

The samples were loaded with 100 µl of a 3mg/ml solution of reconstituted BSA followed by the addition of 200 µl of the specified crosslinking solution. The samples were all sealed via the standard ethyl acetate method and placed in individual glass vials containing PBS pre-warmed to 37 ºC. The vials were then transferred to a heated shaker-water bath set to 37 ºC and 25 RPM. 100% medium changes were conducted every 48 hours, and the release profiles were assessed over 8 days of dissolution via the Bradford-Coomassie method.

7.3 Results & Discussion:

The average cumulative release of BSA (µg) for each formulation was determined and plotted against the 8 days in which the dissolution test took place, and can be seen in Figure 24.

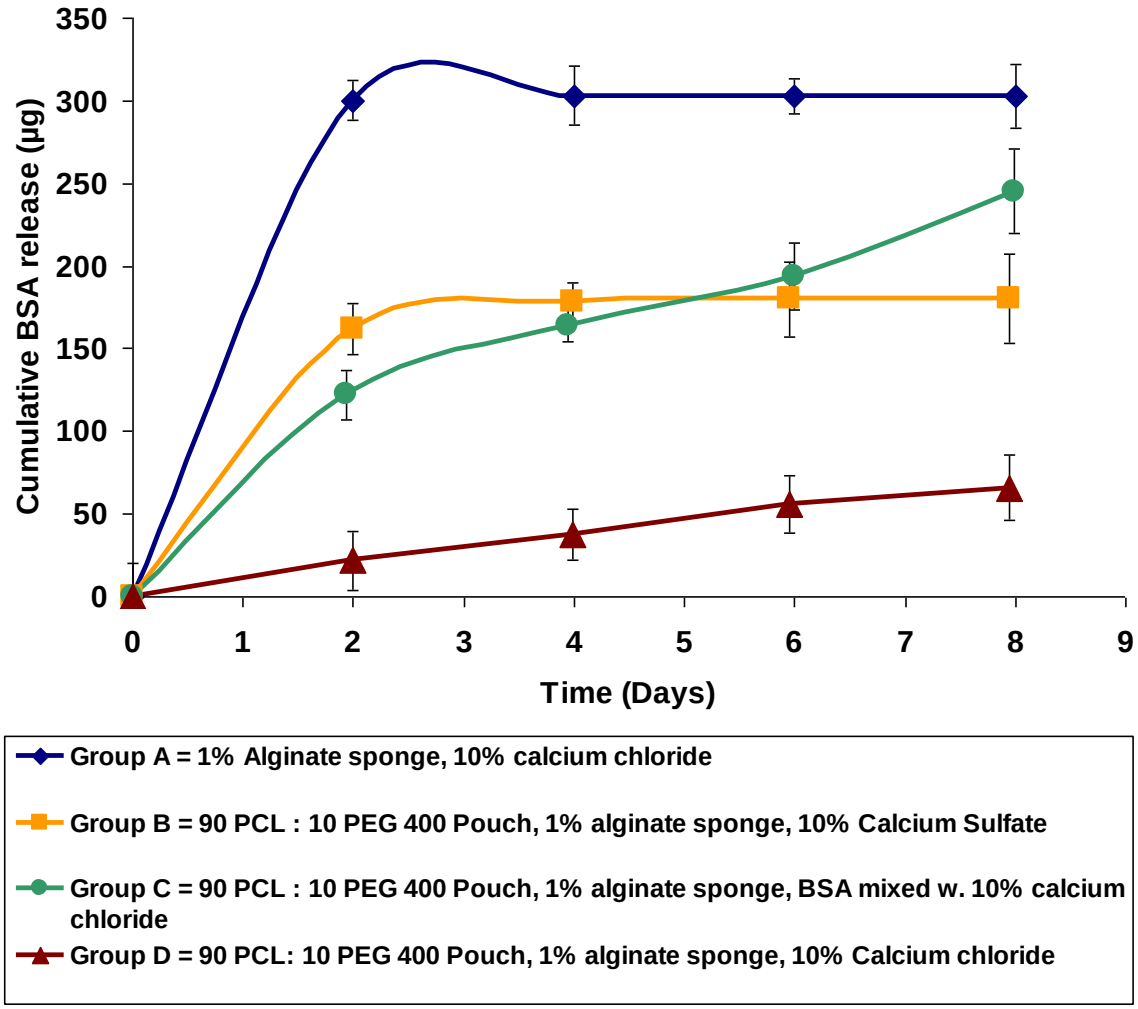


Figure 24: Release characteristics of 90 PCL: 10 PEG 400 Pouches (n=3) with modified crosslinking procedures. A constant loading volume of 300 µg BSA was used for each sample.

It was observed that the formulation with the current crosslinking procedure (Group D) provided the most desirable, low dose release pattern over the dissolution period compared to the other groups, which exhibited burst releases. Group A was expected to have the most rapid release rate of all of the formulations, since the samples within the group did not have the outer 90 PCL: 10 PEG 400 pouch to act as a rate controlling membrane. This proved to be the case, and all 300 ug of BSA eluted out of the pouches within 48 hours. Group B utilized calcium sulfate as the crosslinking agent, which was expected to provide more homogeneous hydrogel networks, therefore a more consistent pattern of release. However, when analyzing the release profiles of Group B compared to group A and B, there was no substantial increase in the consistency of BSA release for Group B. In addition, the release rate was much higher than desired for group B, where a burst release of ~150 µg of BSA was delivered within 48 hours. It was unknown why no more than 150 µg was released over the 8 day period. It was theorized that perhaps due to the slower crosslinking time with calcium sulfate, the 15 minutes allotted for gelation was insufficient to allow complete crosslinking to occur within the alginate. Unsequestered BSA likely contributed to the observed burst release. At the same time, perhaps enough crosslinking occurred within the alginate that approximately half of the BSA remained bound to the alginate past 8 days of dissolution. This would be a plausible explanation, since successful crosslinking with calcium sulfate generally provides slower release rates compared to calcium chloride in alginate based drug delivery systems. Due to the time sensitive nature of orthopedic surgerical procedures, 15 minutes was the maximum time that would be allotted to the in situ preparation of the drug delivery pouch. Since the maximum amount of preparation time was not adequate

to allow for a satisfactory release profile to be generated, calcium sulfate was not considered an acceptable alternative to calcium chloride as a crosslinking agent for the 1% alginate sponge.

Group C was considered a method that had the potential of simplifying the preparation procedure of the drug delivery pouch, since the BSA and calcium chloride would be added to the alginate together instead of separately. The release profile generated by this formulation was both consistent and linear for the most part. however, the formulation shows that the release rate was undesirably high over the dissolution period. Based on the curve, the idea shows promise, and a more intensive experiment with more variations of this formulation will likely be conducted to investigate the idea further.

Based on this study, it was determined that the current crosslinking procedure was the most logical method for crosslinking the alginate sponge. The method allows the drug delivery pouch to provide a linear release profile with a low volume of protein release within the desired range. In addition, the crosslinking procedure is rapid, and falls within the proposed time frame alotted for the in situ preparation of the drug delivery pouch.

Chapter 8: Inherent Viscosity Test for Monitoring PCL Degradation

The two major components of the device's outer pouch consist of 65,000 Mw Polycaprolactone and PEG 400. Though PCL is biodegradable, its material properties, notably its high crystallinity and hydrophobicity, permit a slow rate of biodegradation. In controlled delivery systems, PCL degradation occurs due to hydrolysis, which can lead to open porosity that would influence the rate at which the drug is being delivered [32].

It was hypothesized that during the first 14 days of the delivery period, substantial degradation of the PCL component of the pouch would not occur. An absence of significant degradation would suggest that the release mechanism of the pouch is facilitated solely by initial dissolutions of PEG 400 out of the PCL when placed in an aqueous environment, leaving behind a porous membrane that would allow fluid flow into and out of the device. Fluid transport into and out of the device would induce the subsequent release of encapsulated protein via diffusion. To test this theory, inherent viscosity testing was conducted on the PCL portion of the delivery pouch to monitor its degradation over time.

8.1 Background

Viscosity describes the resistance to flow of a single fluid layer in relation to another layer. As a liquid is moved due to an applied shear stress, at a specific strain rate, the viscosity is defined as the ratio of the applied shear stress to the strain rate resulting from the applied stress [58]. Figure 25 illustrates this phenomenon.

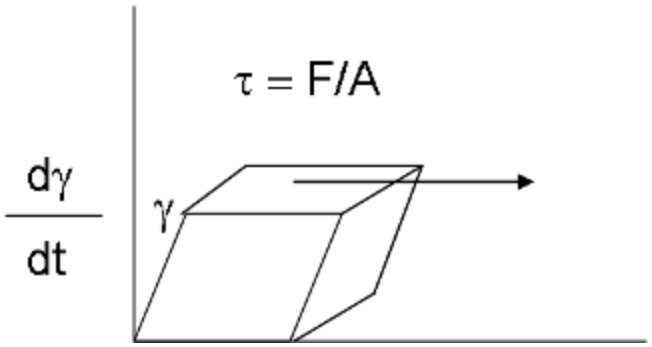


Figure 25: Diagram of shear stress applied to a liquid moving at shear rate dy/dt

In polymer chemistry, the viscosity of a polymer solution can be presented relative to the viscosity of the pure solvent used to dissolve the polymer. Thus, relative viscosity (η_{rel}) is simply the ratio of the viscosity of a polymer solution of known concentration, to the pure solvent that would be used to dissolve the polymer, both determined at equivalent temperatures (Equation 1a and 1b). A simplified equation for relative viscosity of a polymer solution can be seen in Equation 2 [58].

$$\eta_{rel} = \eta/\eta_0 \quad (1a)$$

$$\eta_{rel} = \frac{\rho_1(C_1 t_1 - E_1/t_1^2)}{\rho_0(C_0 t_0 - E_0/t_0^2)} \quad (1b)$$

where:

η = Viscosity of polymer solution (cP)
η_0= Viscosity of pure solvent (cP)
ρ = density (g/cm^3)
c = tube calibration constant (cSt/s)
E = kinetic energy correction constant (cSt·s2)
t = flow time (sec)

$$\eta_{rel} = t_{pol} / t_{solv} \quad (2)$$

where:
t_{pol}= efflux time of polymer solution in viscometer (sec)
t_{solv}= efflux time of solvent in viscometer (sec)

A common, derived function of relative viscosity is inherent viscosity (IV), defined as the quotient the natural log of relative viscosity divided by the concentration of the polymer solution (Equation 3) [59].

$$IV = \frac{\ln \eta_r}{c} \quad (3)$$

where:
η_r = relative viscosity (unitless)
c = concentration of polymer solution (g/dL)

Inherent viscosity is traditionally determined through specialized glass capillary tube viscometers. Figure 26 shows a representative capillary tube viscometer, with the major components emphasized.

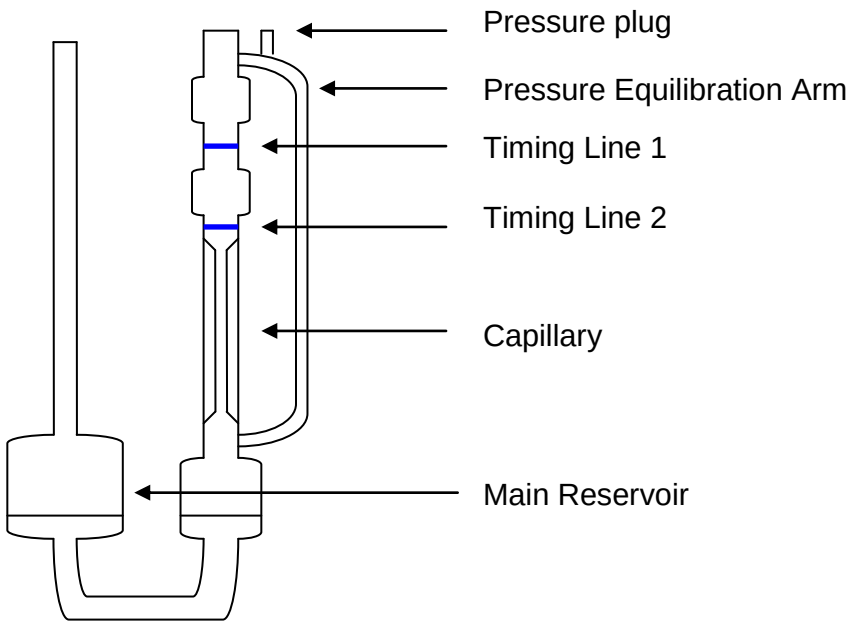


Figure 26: Diagram of a standard type 50 Ubbelohde capillary tube viscometer

The u-shaped glass capillary tube is positioned vertically within a precisely controlled water bath, which in the case of this study, was set at a constant 25.0º C. The reservoir bulb is filled with the polymer solution or pure solvent. Application of suction to the tube mouth draws the liquid up to line 1 on the top test bulb, at this point the suction is terminated, allowing the liquid to flow from the top test bulb down to the lower test bulb. Two etched lines, one situated above the top bulb and one situated below the lower bulb, specify a known volume. The time in which the polymer solution or solvent requires to flow from the top line to the bottom line is recorded during each test, and the efflux time of the polymer solution and the pure solvent are used to determine the inherent viscosity of the polymer.

Polycaprolactone is composed of links of repeating ester groups. A direct relationship exists between IV and polymer molecular weight, in that the higher the molecular weight, the higher the corresponding viscosity of the polymer when dissolved

in solvent. In PCL, chain scission of the ester linkages occurs during degradation which lowers the molecular weight and the corresponding IV value of the polymer [60].

8.2 Methods

To model *in vivo* conditions and their effect on the pouch portion of the delivery device, a single 90 PCL : 10 PEG sheet was fabricated and twelve rectangular 1" x 2" films were cut out of the main sheet. The films were placed in glass vials containing PBS, and the vials were transferred to a shaking water bath set to 25 RPM and 37˚ C. At each time point seen in table 1, three vials (n=3) were taken out of the water bath and the PBS was discarded. Each film was rinsed briefly with DI water for 10 seconds to eliminate any residual PBS that may affect the IV measurement. The films were patted dry with kimwipes, and the films were stored in a vacuum chamber at 2.0 Torr to prevent further degradation until testing was ready to begin. The experiment was run for a total of 16.4 days to determine whether the duration of the proposed drug delivery period would cause a significant change in the film IV.

IV testing was conducted according to the IV measurement specifications outlined in ISO Standard 1628-1, using a calibrated Cannon Ubbelohde viscometer submerged in a circulating water bath set to 25˚C. The viscometer was cleaned with chloroform and dried with nitrogen gas prior to testing and between each sample run. To prepare the samples, the films were individually placed in clean, glass vials and dissolved in chloroform to a concentration of 1 mg/ml.

Testing began with running solvent through the viscometer first to get a baseline reading. 17 ml of chloroform was added to the viscometer, and suction was applied to

draw the solvent into the top test bulb, and the efflux time was recorded. This process was repeated three times, and the two closest solvent efflux times were averaged and used for IV calculations per ISO standard. From there, the aforementioned procedure was conducted for all of the 1 mg/ml sample solutions.

8.3 Results & Discussion

Samples for each time point were tested in triplicate (n=3). The three inherent viscosity values associated with each time point were averaged together and can be seen in Table 5. In addition, the percent change in IV for samples of every time point were compared to the Day 0 samples' average IV value. The plot of the IV values versus dissolution time can be seen in Figure 27.

Table 5: Average inherent viscosity of 90 PCL : 10 PEG films over 16.4 days of modeled dissolution, tested at 25° C (n=3) and percent change in average IV compared to Day 0 samples. Efflux times were measured with a Type 50 Cannon Ubbelohde Viscometer.

Day	Average Film IV (dL/g)	IV Standard Deviation (dL/g)	Percent change from Day 0 IV (%)
0.0	0.9321	0.01200	N/A
2.1	0.9365	0.01080	0.515
7.6	0.9369	0.001618	0.515
11.5	0.9368	0.01413	0.504
16.4	0.9257	0.01078	0.687

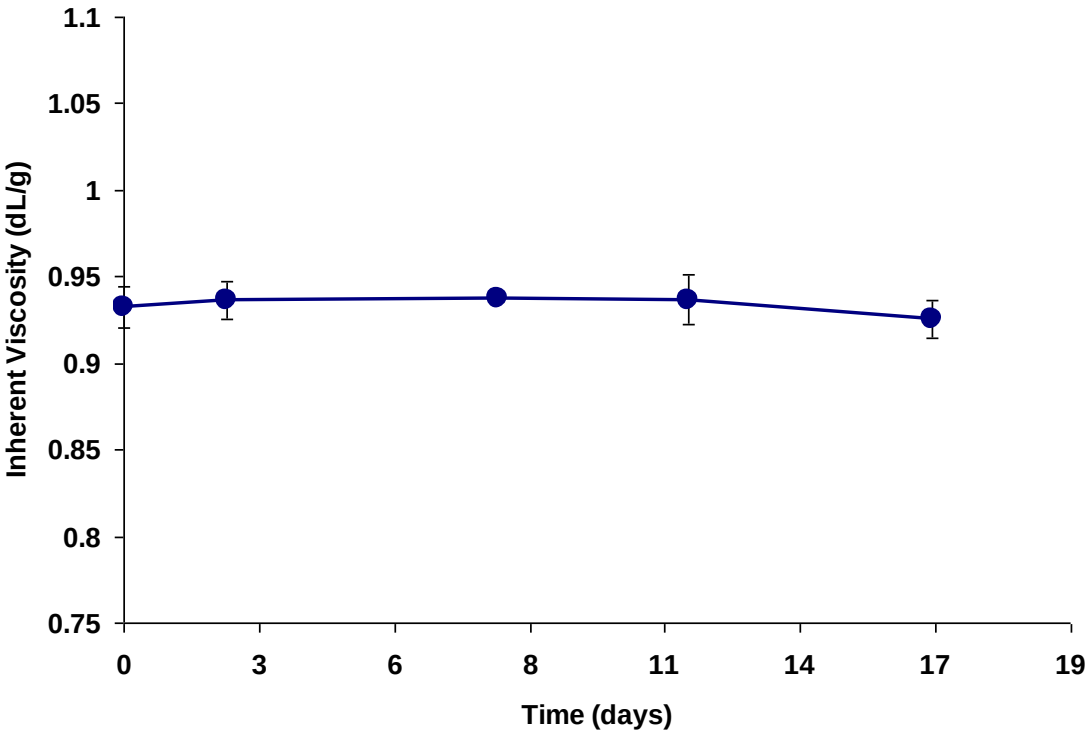


Figure 27: Inherent viscosities of 90 PCL : 10 PEG films over 16.4 days of modeled dissolution.

Over the course of 16.4 days, no significant change in IV was observed for the PCL: PEG 400 films, providing evidence to the theory that very little degradation would occur in the films during this period. Therefore, it was likely that the PEG 400 induced porous network of the PCL was what allowed fluid flow into and out of the device, leading to alginate degradation, and release of encapsulated protein.

In a recent *in vivo* study involving rats implanted with 66,000 MW PCL, full biodegradation occurred over 30 months, which falls in line with the theory [61].

Chapter 9: Characterization of Pouch Release Mechanisms with GPC

No noticeable change in inherent viscosity was observed for the PCL : PEG 400 films submerged in PBS for 16.4 days, suggesting that very limited degradation would occur during the period of protein release. As a verification of this observed trend, GPC was conducted on the same samples in a concurrent experiment and the number average molecular weight and average molecular weight of the films were tracked over 16.4 days of simulated delivery. It was determined that if there was little to no decrease in the Mw and Mn observed over the 16.4 days of submersion, the theory that protein release of the device is facilitated by PEG 400 induced porosity is likely true.

9.1 Background: GPC

Gel permeation chromatography (GPC) is an established size exclusion method used to determine number average molecular weight (Mn), weight average molecular weight (Mw), and molecular weight distribution in samples of dissolved polymers. GPC is often conducted in conjunction with IV testing for the purpose of characterizing polymer degradation rates [62]. The molecular weight distribution of a typical polymer can be seen below in Figure 28, including the areas in which Mw and Mn are generally observed. Shown in Figure 29 is a basic schematic that describes a typical GPC setup.

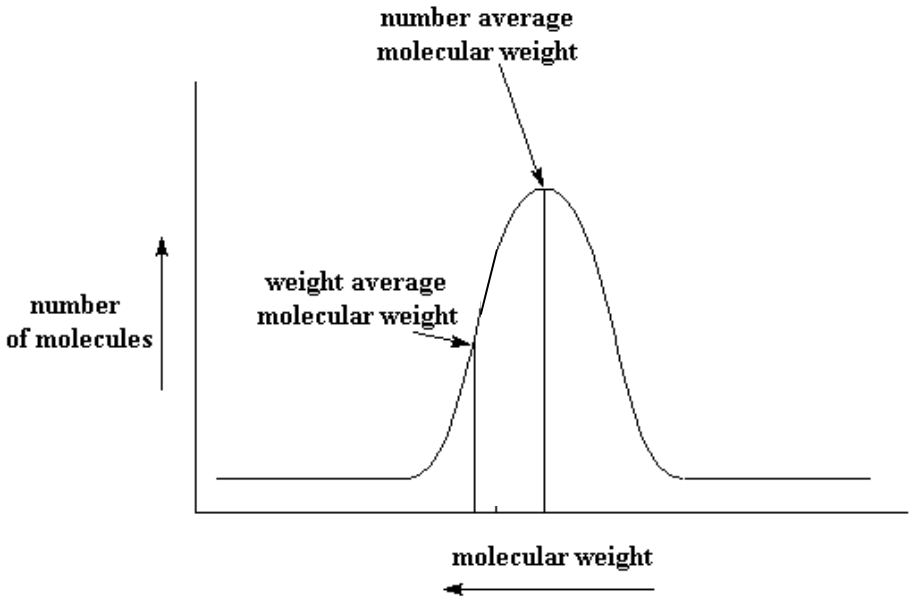


Figure 28: Molecular weight distribution of a typical polymer. [63]

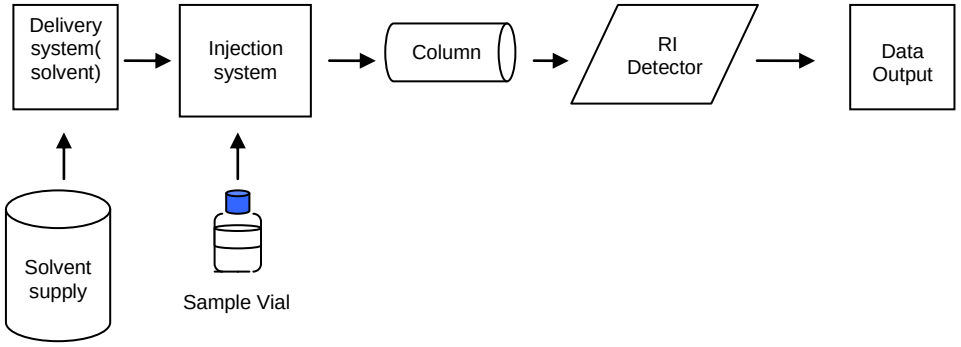


Figure 29: Schematic of a typical GPC Setup

To conduct GPC on a polymer sample, the polymer is first dissolved in a solvent. The sample solution is then injected into the mobile phase, a stream of continuously flowing solvent, the same solvent used to dissolve the polymer. The mobile phase passes through the GPC column (stationary phase), which contains a rigid network of millions of tightly packed silica particles. As the dissolved molecules pass through the particle network, the larger molecules due to their size, pass though the column more rapidly relative to smaller molecules which can enter the particle pores more easily and are therefore retained in the column longer (Figure 30) [64]. The columns themselves are interchangeable and must be chosen based on the material properties of the sample. In addition, the pore size of the particles contained within the columns are well controlled are available in an array of different sizes and efficiencies.

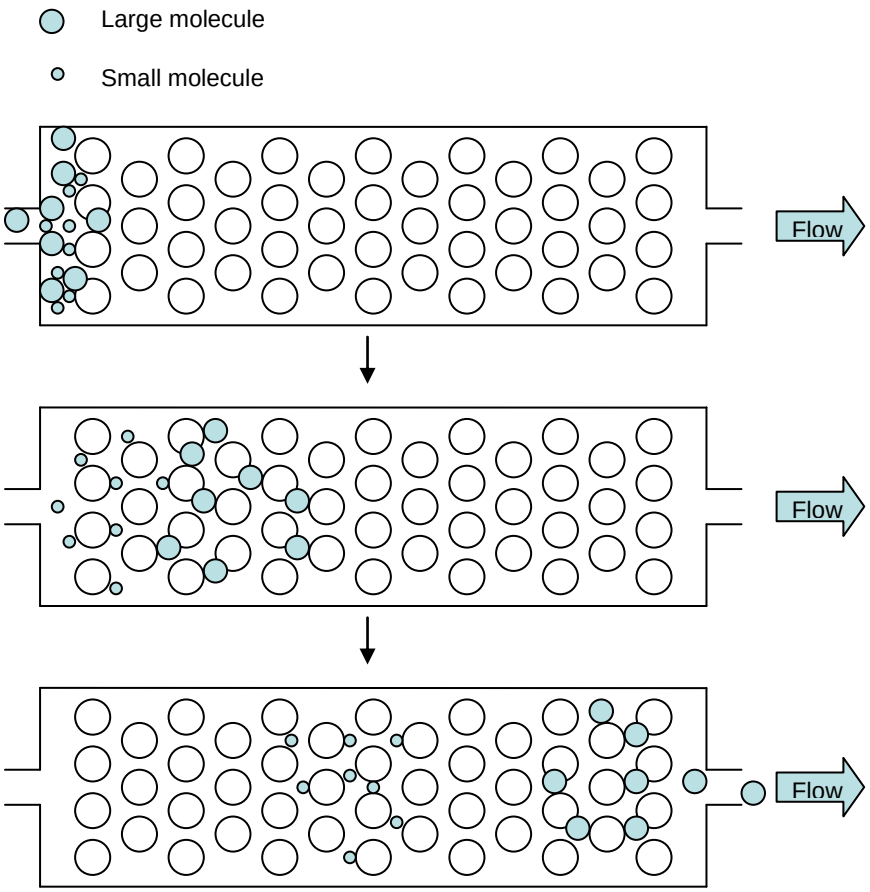


Figure 30: Representation of polymer molecule separation within GPC column based on size.

A detector is positioned at the end of the column that monitors the column eluents. Common detectors are sensitive to concentration, such as those based on UV absorption and refractive index (RI) [65]. The detector in this particular study was RI. The detector and the software package tied to the GPC collaborate to produce a chromatogram and numerical data that can be used for analysis.

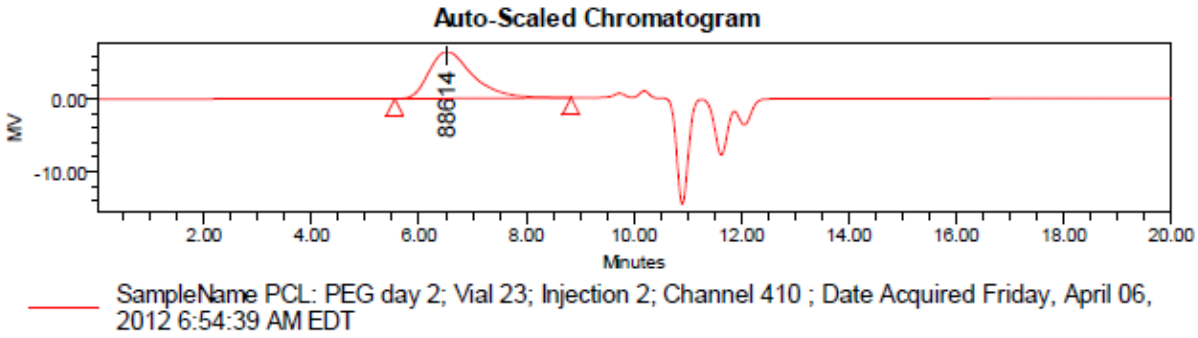
9.2 Methods

The previous study with IV testing involved PCL : PEG films that were submerged in PBS and extracted at various time points up to 16.4 days. Each film was cut in half, and the first half was dedicated to IV. The latter half was used for this GPC study. The PCL : PEG films were dissolved in THF to create solutions at a 1 mg/ml concentration. The solutions were then filtered with 0.45 µm PTFE filters via syringe into pre-slit GPC sample vials. The sample vials and a set of Agilent Technologies GPC calibration standards were loaded into the autosampler tray of a Waters Alliance Separation Unit 2695XE. The system was dry primed and wet primed at a flow rate of 7.5 ml/min for 3 min. A Styragel HR 4E THF 7.6 x 300 mm column was then installed and attached to a Waters 2414 Refractive Index Detector.

The samples were set to run automatically, with 3 injections per sample, and an injection volume of 100 µl. Flow rate was set to 0.25 ml/min. Data acquisition and raw processing was conducted with the Empower software bundled with the GPC.

9.3 Results and Discussion:

A chromatogram for each injection and an organized chart of the results for each time point were produced from the GPC run. A representative chromatogram and table with numerical values can be seen in Figure 31 for a single sample.



GPC Results
Vial: 23

	Vial	SampleName	Name	Injection	Mw	Mn	MP	Polydispersity	Date Acquired
1	23	PCL: PEG day 2	Broad	1	87761	50583	88614	1.73	Friday, April 06, 2012 6:33:13 AM EDT
2	23	PCL: PEG day 2	Broad	2	87757	50385	88614	1.74	Friday, April 06, 2012 6:54:39 AM EDT
3	23	PCL: PEG day 2	Broad	3	87409	50060	88286	1.75	Friday, April 06, 2012 7:16:06 AM EDT
4	23	PCL: PEG day 2	Broad2	1					Friday, April 06, 2012 6:33:13 AM EDT
5	23	PCL: PEG day 2	Broad2	2					Friday, April 06, 2012 6:54:39 AM EDT
6	23	PCL: PEG day 2	Broad2	3					Friday, April 06, 2012 7:16:06 AM EDT

Figure 31: Representative Chromatogram and data output for PCL:PEG films (Day 2)

From analysis of the GPC results, the Mw and Mn determined from each injection were averaged for each time point, and plotted against submersion time (Figure 32 & 33).

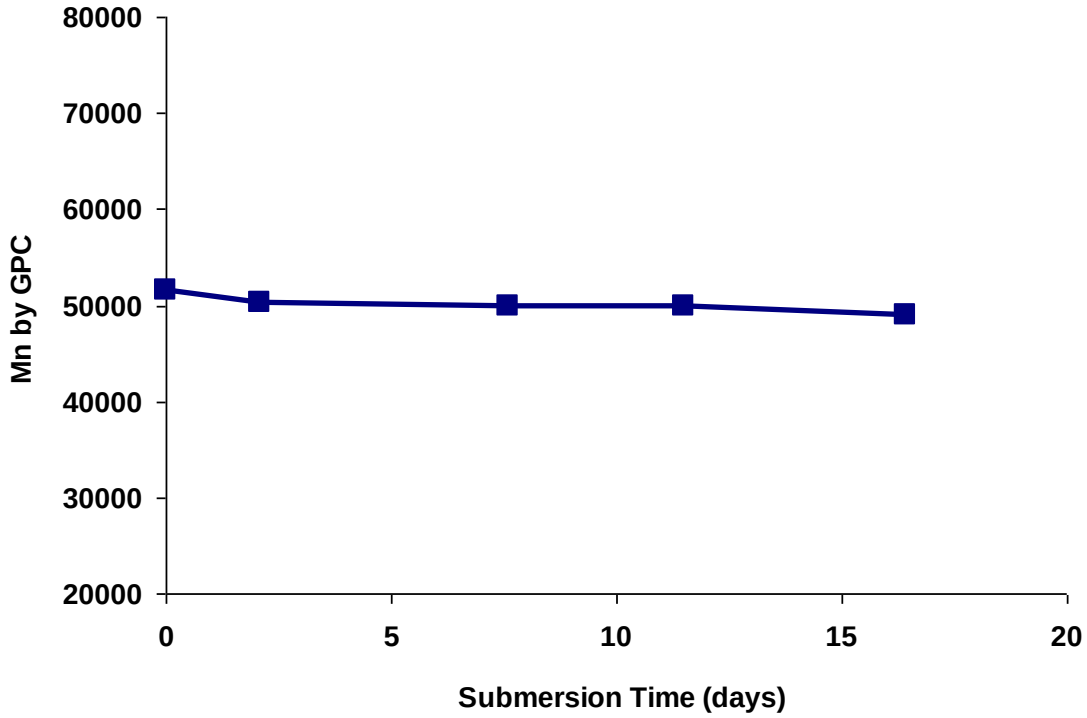


Figure 32: Mn vs. Submersion Time in PBS at 37˚C for 90 PCL : 10 PEG 400 films

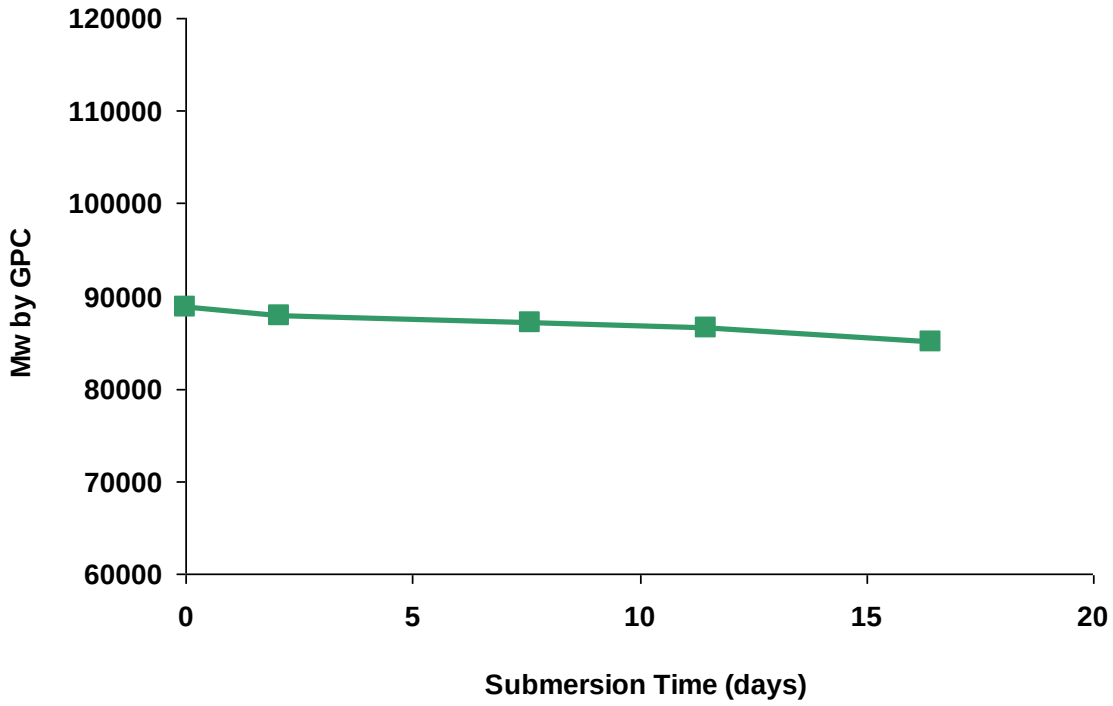


Figure 33: Mw vs. Submersion Time in PBS at 37˚C for 90 PCL : 10 PEG 400 films

By treating the Day 0 sample as a baseline, it was observed that there was a slight drop in Mw and Mn for the film samples from day 0 to day 16.4. The minute changes could be attributed to the inherent molecular weight distribution of the raw PCL material that made up the films, sample variability, and the limited resolution of the GPC column. On the other hand, a decrease in PCL Mw and Mn is indicative of PCL degradation and provides an explanation as to why small amounts of BSA was released after day 8.8 in the 100 PCL : 0 PEG 400 pouches from the earlier BSA release study, even though that particular formulation did not contain PEG 400.

Overall, the same trend of limited to no degradation was observed with GPC testing, which substantiates the IV values obtained previously with the same films. Based on these results, it can be assumed, that the bulk of the release properties of the pouch are attributed to PEG 400 induced porosity.

Chapter 10: SEM Imaging of PCL : PEG 400 Films During Delivery Period

10.1 Background: Scanning Electron Microscopy

Scanning electron microscopy (SEM) is an advanced imaging technique often used in biomedical research to analyze the characteristics of a wide variety of specimens, such as the cell adhesion within an electrospun scaffold or the pore distribution of β-tricalcium phosphate bone substitutes [66].

The components of the SEM are normally housed within a steel column that is placed under vacuum during operation. An electron gun fitted with a tungsten filament cathode is positioned at the top, facing downward. When active, the electron gun emits a beam of electrons onto a condenser lens, which focuses the beam before it passes through pairs of scanning coils positioned within the column. Here, the beam is deflected in a manner that allows a rectangular region of the sample to be scanned. The electrons interact with the sample in a unique matter such as electron deflection (Rutherford Scattering) or loss of energy (inelastic scattering). The electron interactions are monitored by the detector to produce the SEM image. A basic SEM can provide magnifications in the range of 10x to 500,000x and above, much higher than conventional light microscopes [66, 67]. In addition, SEM can provide images of a surface in 3-D instead of 2-D, allowing the user to visualize structures that may not be visible or apparent when viewed under light microscopy [66].

In this study, SEM was utilized to generate images of the delivery pouch material over a two week duration of dissolution, to better understand the structural changes of the

pouch from a visual perspective. With SEM, the pore distribution was assessed, as well as the overall surface and cross-sectional morphologies of the material.

10.2 Methods

10.2.1 Modeling Dissolution

A film of the 90 PCL : 10 PEG formulation was fabricated using the standard method. Four 2.5 cm x 1.0 cm films were cut from the master film, and the first film was considered the day 0 control and would not be subjected to any dissolution. It was placed in a clean glass vial and stored dry in a vacuum chamber set to 7.0 torr until the imaging procedure to prevent any degradation from occurring. The other three films were placed in clean glass vials containing 10 ml of PBS preheated to 37˚ C, and the vials were deposited in a shaking water bath set to 25 RPM and 37˚ C to simulate the effects of the *in vivo* environment. A vial was removed from the water bath at day 2, day 8, and day 14. Upon removal from the water bath, the PBS was discarded from the vials and the films were placed in a vacuum chamber at 7.0 torr to facilitate drying and prevent further degradation of the PCL.

10.2.2 Sample preparation for SEM

4 mm x 4mm squares were cut from the time 0 film. The first square was secured onto the SEM sample peg horizontally so that the PCL:PEG film surface could be observed in the SEM. The second square was subjected to a cryobreak process, which involved submersion of the square in liquid nitrogen for 7 seconds, followed by a break

of the frozen film in half using steel tweezers. The sample was then secured onto an SEM sample peg with the cross section facing upwards so that the cross section could be analyzed with SEM. The samples were then sputter coated with gold powder using a Denton Desk IV HP sputtering system, to enhance the SEM image contrast. Image acquisition and processing was conducted with the Hitachi software bundled with the SEM.

10.3 Results & Discussion

The SEM images obtained from this study are shown in Figure 34 and are organized into the top views of the films and the cross sections of the films in descending order.

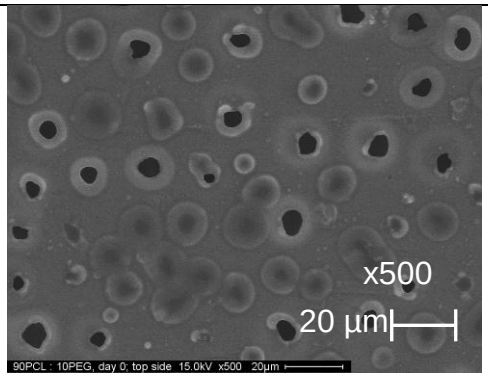
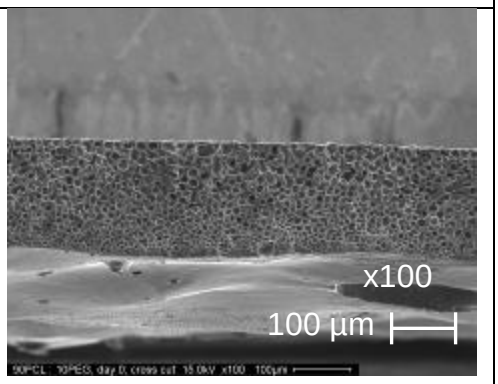
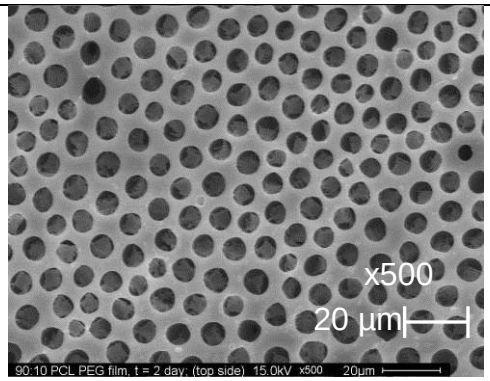
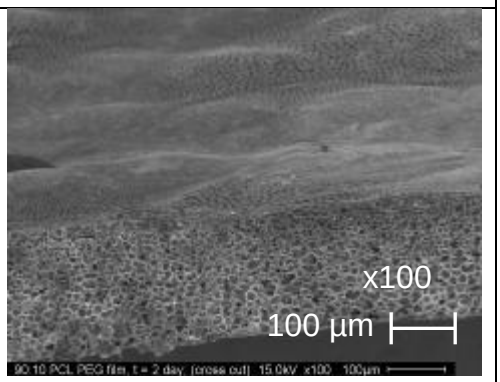
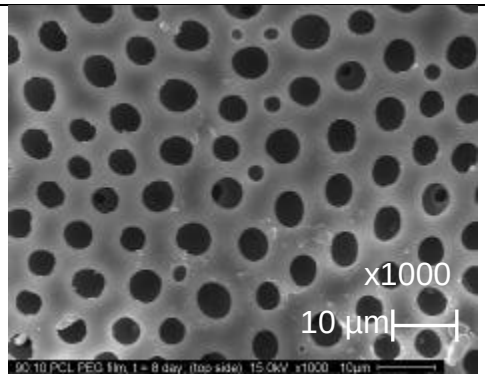
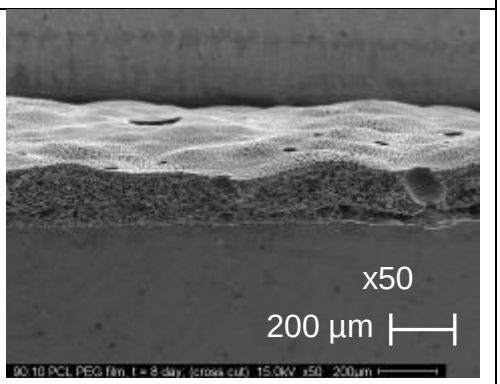
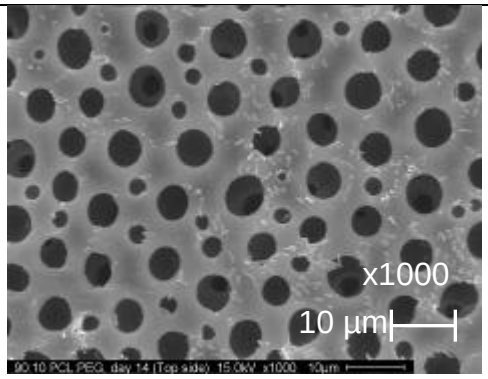
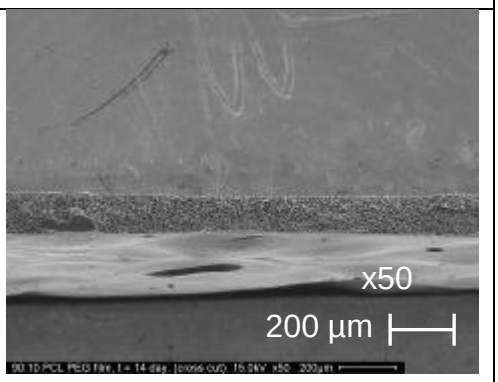
Submersion Time in PBS (pH 7.4 & 37°C)	90 PCL : 10 PEG 400 film (Top View)	90 PCL : 10 PEG 400 film (Cross-Sectional View)
Day 0		
Day 2		
Day 8		
Day 14		

Figure 34: SEM images of 90 PCL : 10 PEG 400 films after submersion in PBS for up to 14 days

From the Day 0 image, it was observed that the PEG 400 was impregnated within the PCL during the blending process, forming evenly distributed bulbous regions of accumulated PEG 400 on the PCL film surface. It was interesting to observe that even at day 0, a small amount of the PEG 400 had migrated away from the film, leaving behind a few open pores where there were originally bulbous portions of accumulated PEG 400. In the day 2 film, the bulbous regions of PEG 400 that were present at day 0 are absent, and instead, a homogeneous array of open pores, approximately 2-5 μm in diameter, were present over the surface of the film. This suggested that all of the PEG 400 dissolved out of the film within 2 days of submersion in PBS, leaving behind just the porous PCL film. PEG 400 is soluble in aqueous media, while PCL is substantially more hydrophobic, so it is likely that the PEG 400 was completely gone from the PCL earlier than 2 days. The films for day 8 and day 14 are very similar to the day 2 film in terms of pore size and distribution. However, at day 8, a few additional pores were observed in the PCL film that had begun to form, that are slightly smaller in diameter (~1-3 μm) compared to the original pores. On day 14, more of these small pores are present. It is likely that the new pores formed due to the minute amounts of degradation in the PCL film during the submersion period.

The cross section of the day 0 sample showed how the pore network is distributed throughout the film prior to placement in a simulated physiological environment. This same pore network is seen in the day 2, day 8, and day 14 samples as well. It is likely

that the pore network is the major facilitator of protein transport from within the drug delivery pouch to the outside environment during the duration of the delivery period.

Chapter 11: Determination of % Loss of PEG 400 from 90 PCL:PEG 400 films during Dissolution Period

11.1 Background:

The dissolution of PEG 400 is an important aspect of the device design, since it directly contributes to the release profile of the device. From the SEM images, it was observed after submerging the films that the PEG 400 would dissolve out within a 48 hour period. However, it was unknown exactly how long it would take within that 48 hour period for the PEG 400 to elute out. To characterize the device further, the objective of this study was to quantitatively determine how long PEG 400 is retained in the film after it is submerged.

To accomplish the experimental goal, the mass of the films were taken into account. Based on the IV and GPC data, very little degradation of the PCL occurred during the two week release period. For this reason, it was theorized that any mass lost in the PCL: PEG 400 film after it is submerged in aqueous media should be attributed to the dissolution of PEG 400 alone, rather than PCL degradation during the first 48 hours.

In this study, the mass change of several PCL: PEG 400 films after submersion in DI water were monitored over 48 hours, to calculate how long PEG 400 remained in the films after they are placed in media.

11.2 Methods

A 90 PCL : 10 PEG 400 film was fabricated using the standard method. Eight rectangular films approximately 2” x 1” were cut from the main film and pre-weighed. These films were then transferred to glass vials containing DI water preheated to 37 ˚C,

and the vials were placed in a shaking water bath set to 37 ˚C and 25 RPM. A single vial was extracted predetermined timepoints up to 48 hours. The films were isolated after extraction and placed under vacuum at 7.0 mmHg for 24 hours which would remove any residual water within the films while allowing any leftover PEG 400 to be retained. The vapor pressure for peg 400 is less than 0.01 mmHg at 20˚ C while the vapor pressure for water at 20˚ C is 17.3 mmHg so the pressure was low enough to extract the water but not PEG 400.

The films were weighed again to calculate the change in mass of the films after submersion in DI water. As a note, DI water was chosen as the media instead of PBS, due to the fact that any residual PBS would form salt crystals on the sample films during the vacuum drying procedure and add extra mass. Rinsing the films to remove residual PBS would not be feasible either, since the process would likely rinse off the PEG 400 in the film in addition to the PBS, possibly skewing the results.

The standard 90 PCL:10 PEG 400 film is normally cast using a homogeneous mixture of 9 ml of a 10% PCL solution and 1 ml of PEG 400. The mass of PEG 400 and mass of PCL that comprise a single 90 PCL: 10 PEG 400 film were determined as follows: To find the mass of the PCL component, a film was cast with 9 ml of a 10% PCL/chloroform solution alone, without the 1 ml of PEG 400. The film was then weighed after the solvent evaporation process. To obtain the mass of the PEG 400 component, 1 ml of PEG 400 was deposited on a tared aluminum pan and the mass was recorded.

A representative calculation for a sample film can be seen below in Figure 35. These calculations were conducted for each of the samples that were included in the experiment and the values can be seen in Table 7.

Initial mass of sample film (prior to submersion) = Mi = 0.0902 mg
Final Mass of sample film (after 15 min of submersion) = Mf = 0.0420 mg

Total PEG content in sample film = (Initial mass of film sample) x (mass of PEG component in full film)/(mass of full film)
= (90.2 mg) x (1g /1000 mg) x (1.0816g) /(0.82998g +1.0816 g) x (1000 mg/1 g)
= 51.0 mg of PEG 400

% PEG 400 lost from film sample = (mass change of film sample) / (total PEG content of sample) * 100
= [(48.2 mg)/ (51.0 mg)] x 100
= 94.4 % PEG lost

Figure 35: Representative Calculation for Sample L3

11.3 Results & Discussion:

With the values obtained during the experiment, dimensional analysis was used to calculate the total PEG 400 content of each film sample, as well as the % PEG 400 lost from the sample films at each time point, which can be seen in Table 6 and Table 7.

Table 6: Mass of individual components of a 90 PCL : 10 PEG 400 film

Mass of PCL film cast from 9 ml of 10% PCL/chloroform solution (g)	Mass of 1 ml of PEG 400 (g)
0.82998	1.0816

Table 7: Experimental values obtained from film samples submerged from 0 – 48 hours. These values were used to determine % PEG 400 loss over time.

Sample Name	sample film submersion time (hours)	initial sample film weight (mg)	final sample film weight (mg)	mass change of sample film (mg)	percent mass change of sample film (%)	Total PEG 400 content in sample film (mg)	PEG 400 lost from sample film (%)
L1	0.00	76.1	76.1	0.0	0.0	43.1	0.0
L2	0.02	57.5	28.1	29.4	51.1	32.5	90.4
L3	0.25	90.2	42.0	48.2	53.4	51.0	94.4
L4	1.00	72.3	32.1	40.2	55.6	40.9	98.3
L5	5.00	140.7	60.3	80.4	57.1	79.6	101.0
L6	12.00	72.2	31.3	40.9	56.6	40.9	100.1
L7	24.00	90.8	39.1	51.7	56.9	51.4	100.6
L8	48.00	125.2	53.0	72.2	57.7	70.8	101.9

The % PEG 400 lost from the sample films versus the corresponding submersion time were plotted and can be seen in Figure 36.

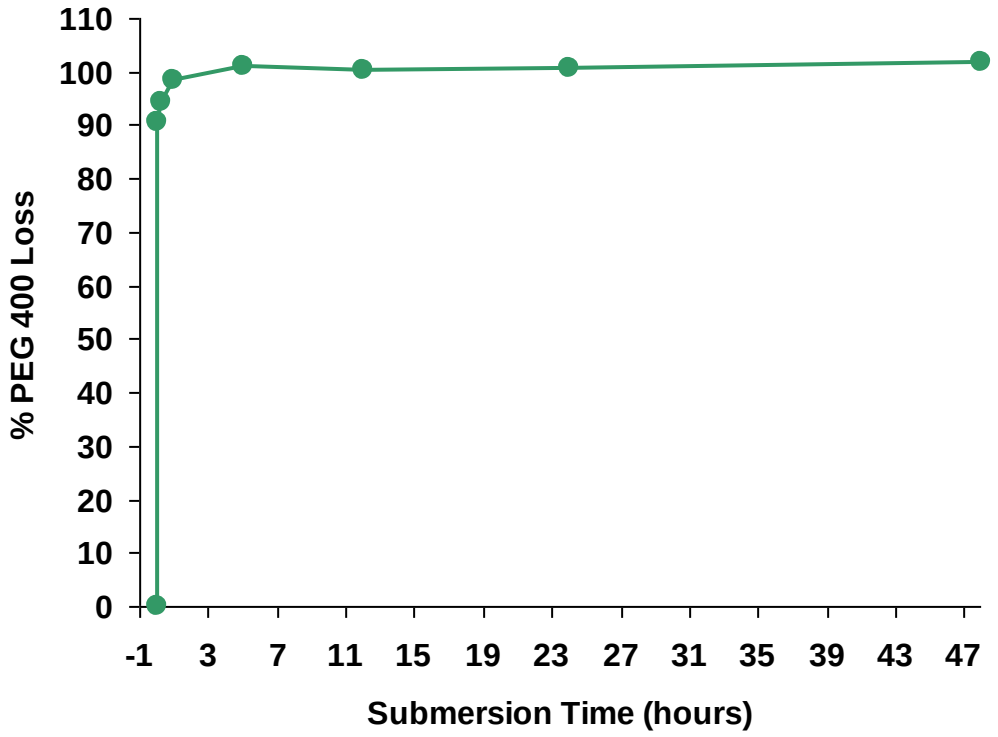


Figure 36: Graph of % PEG 400 lost from sample films over a 48 hour period of submersion in DI water.

It was observed that the films lost 90.4 % within 1 minute, 98.3 % within 1 hour, and ~100% of the total PEG 400 content within 5 hours, which quantitatively substantiates the theory that the PEG 400 would dissolve out of the films very quickly upon submersion in water. As a note, the % PEG 400 lost for the latter time points were very close to, but not exactly 100 %, which were attributed to experimental error.

Overall, the phenomenon observed in this study was desirable from a design standpoint, and shows how the PEG 400 was a solid choice for the rate controlling membrane portion of the drug delivery device. It allowed for a predictable way to

modulate the release profile of the pouch as seen in Chapter 6. In addition, it helps facilitate immediate release of BSA in aqueous media, but at the same time, the pores created from the PEG 400 are small enough to prevent the occurrence of an undesirable burst release.

As a supplementary observation, the total PEG 400 content of the 90 PCL : 10 PEG 400 films was ~57% by mass. When analyzing the SEM images of the 90 PCL : 10 PEG 400 films, the porosity was estimated to be about 50-55 %, which correlates well with the findings of this study.

Chapter 12: Preparation for Bioactivity Testing with Enzyme-linked Immunosorbent Assay (ELISA)

12.1 Background: Protein Quantification and Bioactivity Determination with ELISA

The enzyme-linked immunosorbent assay (ELISA) is an analytical microtiter plate-based test used in medical diagnostics, quality control, and biological research for the quantitative detection of specific proteins [68]. There are several types of ELISAs available for various applications, with the sandwich ELISA being the most common. A competitive ELISA was used in this preliminary study, which has the advantage of high specificity, minimal sample processing, and less sensitivity to sample dilution compared to the standard sandwich ELISA [69].

The current parameters applied in the pouch fabrication and loading demonstrate that the pouch design can release the model protein, bovine serum albumin, with the desired delivery profile. The next logical step in development would involve testing the device's compatibility with BMP-2. A standard sandwich ELISA was planned to fulfill two experimental objectives: (1) verify that the BMP-2 tertiary structure is maintained over the 2 week release period for the device, and (2) determine the release profile of BMP-2 from the delivery pouch.

ELISA requires site specific binding of the target protein to the target antibody coating the immunoplate. Since a protein's structure and its overall function are closely related, the ELISA can be used in part to determine whether the BMP-2 delivered from a controlled delivery device can still bind to the BMP-2 specific antibodies present on the ELISA immunoplate. Successful binding would allow for the ELISA to produce a visible

signal, which would then suggest that the BMP-2 released from the device is still functional [70]. In addition the signal produced from the ELISA can be converted to a protein concentration reading, which could be used to determine the release profile of BMP-2 from the delivery pouch [68, 71].

Due to the high cost of BMP-2 required for the ELISA and other tests associated with BMP-2 bioactivity, a BSA specific ELISA was conducted first as a preliminary test, with the intent that if the results showed promise, the BMP-2 ELISA would be conducted afterward.

In this study, standard delivery pouches were fabricated and loaded with varying amounts of BSA. The pouches were then placed in dissolution conditions, and the BSA ELISA was conducted on the release medium, to determine if a visible signal could be generated. The visible signal would suggest that the BSA remained structurally stable during the entire duration of the release period, and that the device, when paired with BMP-2, would likely deliver BMP-2 without structural damage as well. In addition, release profiles for the different pouch formulations were calculated based on the concentration readings generated by the ELISA. Since three different loading doses were used for each sample group, it was expected that the higher loading dosages would correspond with higher amounts of cumulative BSA release.

12.2.1 Binding Reactions involved in Competitive ELISA

The general competitive ELISA procedure consists of several steps:

1. Incubating a known amount of primary antibody with the sample (antigen) containing an unknown amount of protein. In solution, the primary antibodies and antigens will bind to form complexes [72].
2. This solution is then added to the microtiter plate wells that would be pre-coated with the antigen. Any unbound primary antibody will bind to the protein coated wells, and thus compete for available binding sites [72].
3. For signal generation, the plate is first thoroughly washed with wash buffer to remove unbound antibody. Goat-anti-rabbit-conjugated horseradish peroxidase (Go-a-Ra-HRP or HRP) is added to the plate, which will bind to the remaining antibodies immobilized on the plate [72].
4. Lastly, following a wash to remove any residual, unbound HRP, 3 3' 5 5'-tetramethylbenzidine dihydrochloride (TMB) will be added to the plate, which will react with the bound HRP to produce a visible color change. The intensity of the color development is linearly related to the amount of antibody that is bound to the plate, so in effect, the higher the concentration of antigen in the sample, the less intense the resultant signal will be due to the competitive nature of the assay [72]. The aforementioned steps are illustrated in Figure 37.

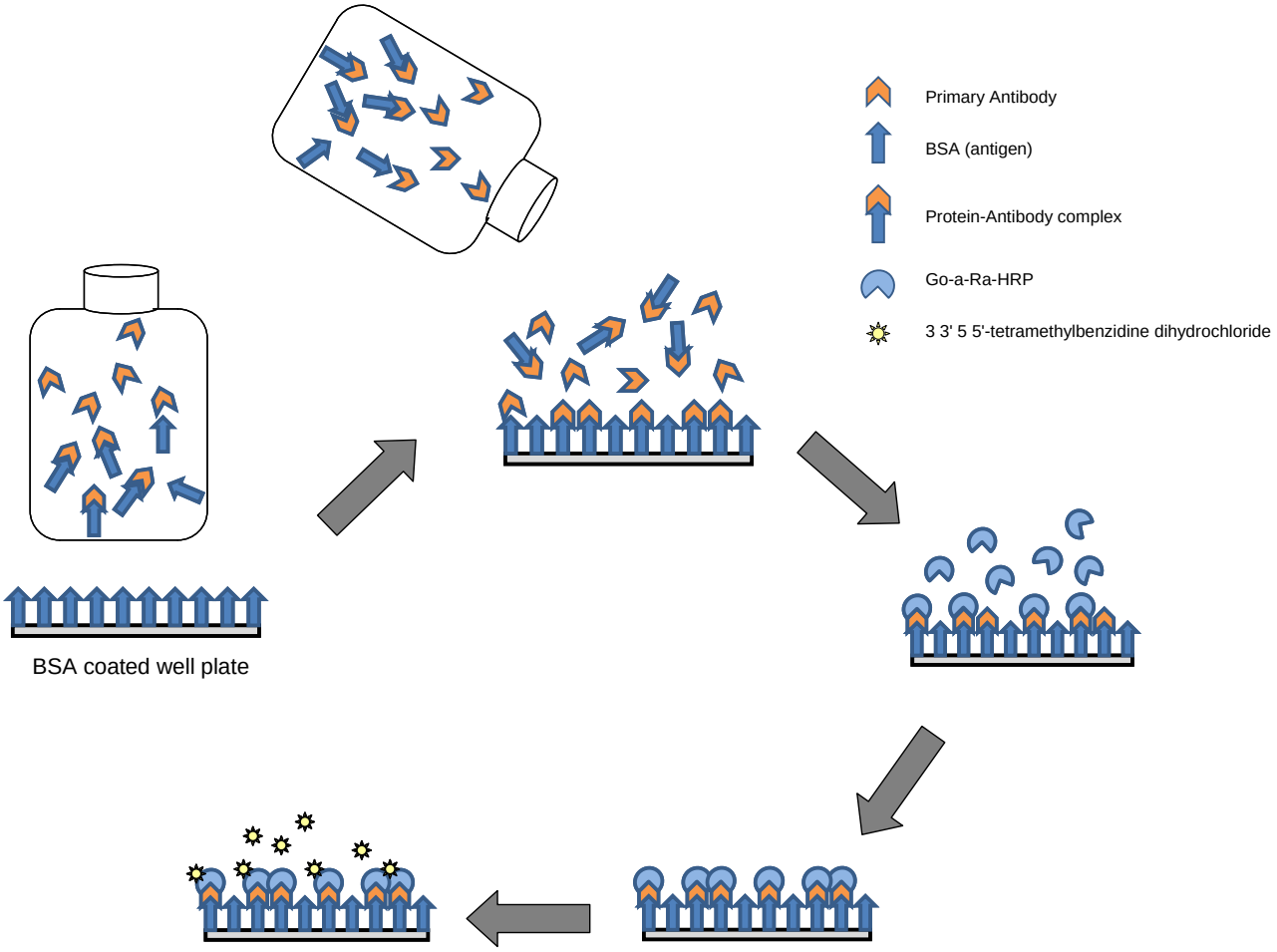


Figure 37: Diagram depicting binding reactions involved in the competitive ELISA procedure

12.3 Methods

Following the scheme represented in Table 8, Six 90 PCL: 10 PEG 400 pouches were fabricated using the standard method. As a note, the scheme was designed to utilize all 96 wells of a single ELISA plate so instead of running the experiment in triplicate, the samples had to be prepared in duplicate instead. The required amount of BSA solution was loaded into the 1% alginate sponges, and the sponges were crosslinked using the standard 10% $CaCl_2$ method. In preparation for the BMP-2 specific ELISA, three BSA

dosages much lower than the standard 3 mg/ml were chosen for loading the pouches, since the BMP-2 specific ELISA had an extremely low detection range of 0-4,000 pg/ml.

Table 8: Sample Preparation Outline for Dissolution testing with BSA ELISA

number of samples	loading dose of BSA (µg) contained in 100 µl of DI water	concentration of loading dose of BSA (mg/ml)
n=2	100	1
n=2	10	0.1
n=2	1	0.01

The individual pouches were placed in glass vials and 10 ml of PBS prewarmed to 37˚C was added to each of the vials. The vials were placed in a shaking water bath set to 37˚C and 25 RPM. 100% of the dissolution media within the vials was replaced every 2 days, and the old dissolution media was stored at 4 ˚C until the assay was ready to be conducted.

The BSA competitive ELISA was purchased from Bachem Holding AG and prepared according to the included protocol. Briefly, 75 µl of sample/standard was mixed with 75 µl of primary antibody in a clean 96 well plate and left to incubate for 1 hour at room temperature (23˚C). The solutions in each well were transferred to the BSA-coated immunoplate. The immunoplate was incubated for 1 hour, followed by a thorough wash with the provided wash buffer. HRP was added to each well, followed by an additional 1 hour incubation period and a second wash with wash buffer. Lastly, TMB was added to each well, and the plate was incubated for 1 hour. Stop solution (2 N HCl) was added to each well after a significant level of color development was established and

the immunoplate was read at 450 nm using a PerkenElmer Victor3 plate reader (Figure 38). The standards and all samples were assayed twice on the same ELISA plate, per the established method [72].

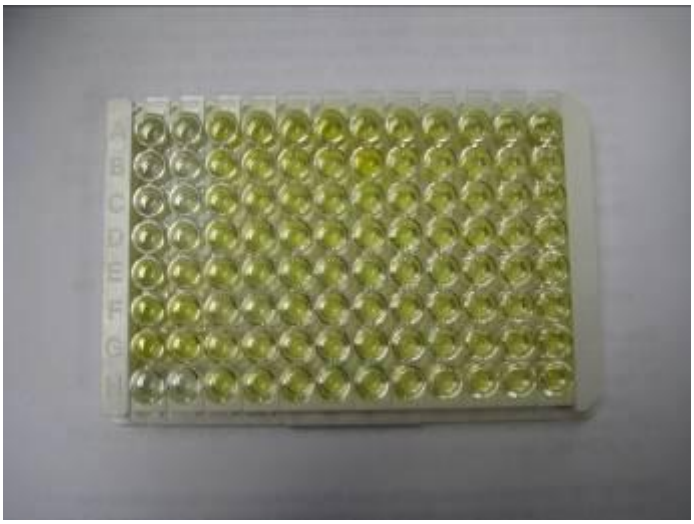


Figure 38: Completed BSA ELISA Immunoplate following reaction termination with 2N HCl stop solution.

12.4 Results & Discussion

Shown in Figure 39 are the dissolution results of the three pouch formulations, determined from dissolution testing and analysis of the competitive ELISA readings.

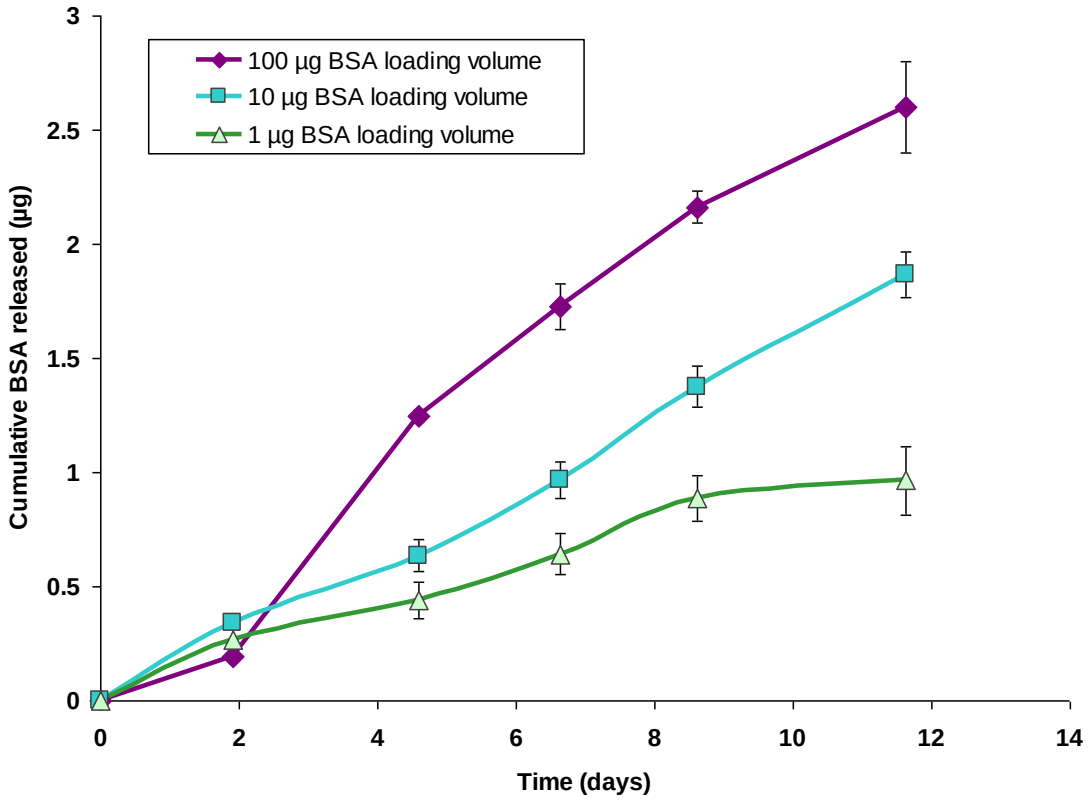


Figure 39: Release characteristics of 90 PCL: 10 PEG 400 Pouches with varying loading dosages (n=2).

The ELISA results showed controlled release from all three pouch formulations, thereby verifying that a complex protein could be successfully loaded into the delivery pouch and released in a consistent, predictable manner. Since a visible signal was attained while reading the ELISA immunoplate, it was determined that the tertiary structure of the BSA was uncompromised during the delivery period, to the point where the BSA binding sites specific to the ELISA were still functional after 2 weeks. It was expected that with increasing BSA loading dosages, there would be a corresponding

increase in the release of BSA over time, which was observed. Overall the results of this test were very encouraging and compromised tertiary structure or diminished bioactivity was not expected for pouches loaded with BMP-2.

Chapter 13: Conclusions

Over the course of the design project, a novel, controlled protein delivery device was developed with regard to several specific aims and design criteria/constraints. Extensive patent and literature searches were conducted to ensure that the implant was unique and unlike anything available in the current market, and a series of tests were conducted to verify that the design performed as desired.

Specific Aim 1 was fulfilled in that the device was indeed a dual component drug delivery system comprised of (1) a drug reservoir that also acts as a platform for controlled release and (2) a vial of lyophilized model protein which can be reconstituted and loaded into the drug reservoir at the time of use. In addition, the pouch design allowed for successful loading with reconstituted BSA within the desired 20 minutes, thereby fulfilling Specific Aim 2.

Several pouch formulations with varying ratios of PCL and PEG 400 were capable of providing controlled protein release in the 1.37-25.7 μg range over at least 14 days, with no burst release, fulfilling the requirements of Specific Aim 3.

Lastly, Specific Aim 4 was fulfilled. The release characteristics of the device, as well as alternative preparation methods and their effect on the pouch release rate were investigated. In addition to the protein delivery aspects, the material degradation and surface morphologies of the drug reservoir were monitored with respect to physiological conditions to understand this novel technology to the degree that future modifications to the design could be conducted if necessary.

Unfortunately, Specific Aim 5 was not fulfilled. In depth methods for conducting a BMP-2 specific ELISA and the Alkaline Phosphatase assay were developed to assess

the bioactivity of the BMP-2 released from the device, which can be seen in Appendix A, but due to time and cost constraints, the test was postponed for an indefinite period. However, completion of the preliminary BSA specific competitive ELISA verified that a complex, reconstituted protein could be successfully loaded into the device and released in a controlled manner. The protein structure and suggested functionality of the BSA was retained during and after delivery to the point where the binding sites were still active after ~ 2 weeks. These results were quite promising, and diminished bioactivity was not expected to occur in future tests involving pouches loaded with BMP-2.

Chapter 14: Recommendations

Future work for this project should involve cell based testing with reconstituted BMP-2 in addition to the BMP-2 specific ELISA mentioned previously. A preliminary assay was developed to determine the potential of the delivery pouch in its ability to stimulate alkaline phosphatase activity in C2C12 cells over a two week duration of controlled protein release (Appendix B) and likely will be conducted in the future when more funding is available.

Other types of biomaterials should be assessed as possible alternatives to the materials used to create the pouch. For example, chitosan, a naturally derived biomaterial offers advantages such as biodegradability, established biocompatibility, and bioadhesive properties. Chitosan can also be processed in a wide variety of ways, to create stable hydrogels, providing a feasible alternative to sodium alginate [51].

The concentration of sodium alginate used to fabricate the sponge portion of the delivery pouch was kept constant at 1% w/v for the duration of this project. In the future, variations in the initial alginate concentration should be tested to determine its effect on the release profile of the device.

Farther in the future, preliminary in vivo testing should be conducted with a rat tibial defect model to help ensure that the BMP-2 delivery profile of the device is both effective and safe enough for further development involving larger animal models as well as human patients.

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Appendix

Appendix A: Dissolution and Bioactivity test for BMP-2 Pouch

Methods:

1. Construct (3) pouches following the diagram presented in figure 1.

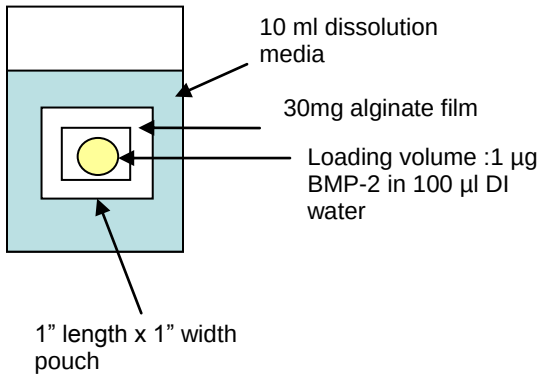

Figure A-1: Pouch dimensions and dissolution scheme for ELISA

2. 1 µg of BMP-2 will be required per pouch:
    a. Add 100 µl of DI water to (6)1ug vials of BMP-2 and mix gently to reconstitute the BMP-2. Leave the solutions in the refrigerator at 4˚C until ready to use. Do not vortex.
3. Add the full contents of two 25 ug vials to each pouch.
4. Add 200 ul of 10% $CaCl_2$ to each pouch to crosslink the hydrogel component. Let stand for 5 minutes.

5. Pour out excess liquid and seal each pouch with ethyl acetate and a metallic scraper.
6. Place each sealed pouch in a 20 ml scintillation vial and add 10 ml of cell culture media. Since C2C12 cells will be used for testing later on, use growth medium suitable for C2C12 cells:
    a. DMEM (GIBCO # 11965) = 490 ml
    b. FBS (Hyclone # 30071.03) = 10 ml
    c. 1X penicillin/streptomycin = 0* ml

    500 ml total volume
7. Place the vials in a shaking water bath at 25 RPM and 37°C.
8. After 48 hours of dissolution, replace 100% of the dissolution media. For sampling, obtain 0.25 ml of the dissolution media and dilute in 99.75 ml of fresh PBS.
9. Take samples every 48 hours over the course of 14 days (total of 8 readings per pouch). Store samples in the refrigerator until ready for use in the ELISA.
10. conduct the ELISA per instructions provided with R&D Systems: Quantikine BMP-2 ImmunoAssay.

Appendix B: Alkaline Phosphatase Assay for Delivery Pouch

Background:

The purpose of this experiment is to assess the potential of the BMP-2 pouch in its ability to stimulate alkaline phosphatase activity in C2C12 cells over a 14 day duration of protein release.

Methods:

Growth Medium:

1. DMEM (GIBCO # 11965) = 490 ml
2. FBS (Hyclone # 30071.03) = 5 ml
3. 1X penicillin/streptomycin = 5 ml

500 ml total volume

Procedure for preparing C2C12 cells from frozen stock:

1. Remove cryovial containing the C2C12 cells from liquid nitrogen and place in 37ºC water bath. Gently swirl the cryovial and thaw cells quickly within 1 minute.
2. Wipe off the vial with 70% ethanol and transfer the vial to the laminar flow hood.
3. Transfer cells into a 50 ml conical tube containing 30 ml of prewarmed growth medium.

4. mix gently and transfer the cell solutions (10 mls) to two T-75 flasks. Incubate flask at 37ºC and 5% CO2
5. Replace media the next day and assess cells under microscope to verify cell adherence.

Cell passage:

1. assess cell density under inverted microscope. Cells are ready to split at 60 % confluence.
2. Prewarm culture medium, Trypsin-EDTA, PBS in 37ºC water bath. Spray down with 70% ethanol prior to transferring to hood.
3. Remove old cell culture medium from flask. Wash cells once with PBS (5 ml), rock gently, and aspirate the PBS.
4. add 2 ml Trypsin-EDTA solution to flask and place in incubator for 3 minutes. observe cells using inverted microscope to assess detachment. This should occur within 5 – 15 minutes.
5. Resuspend cells by adding 8 ml of culture medium so that a total of 10 ml of solution is contained within the flask. Rinse the sides of the flask to maximize the number of cells harvested. Count the cells using a hemacytometer
6. split the cells 1:4
    a. prepare two new T75 flasks by adding 7.5 ml of fresh culture medium
    b. Add 2.5 ml of cell suspension to each flask.
7. incubate flask at 37ºC and 5% CO2

Pouch elution:

1. Construct the pouch and sponge components using the standard method. Verify that the loading port is the correct size for sealing in the hood. Place all pouches that are to be used for the experiment in a 50 ml centrifuge tube containing 70% ethanol for 15 minutes.
2. Extract pouch and sponge components in the hood and allow them to dry for 1 hour. Alternatively, unscrew the centrifuge tube partially and place in the vacuum oven for 1 hour to extract all water and ethanol.
3. bmp-2 stock solution preparation
    a. weigh out
4. Filter sterilize with 0.2 um filter…0.22 um filters composed of cellulose acetate, polypropylene, or polyether sulfone for maximum protein retention.

5. create a 50 ml 10% solution of calcium chloride and filter sterilize it with 0.22 um filter
6. obtain 6 sterile 15 ml conical tubes.
    a. Add 2.5 ml of cell culture media to the conical tube. (aim for infinite sink conditions x 3)
    b. Following the loading scheme outlined below
        i. load each pouch with the appropriate volume of bmp-2 and place in the 15 ml sterile conical tubes.

Bmp-2 loaded pouch	BMP-2 loaded pouch	BMP-2 loaded pouch
unloaded pouch	BMP-2 alone (control)	Cells only (control)

c. Place the tubes in a shaking water bath at 37ºC

d. Change media every other day. Keep samples in the refrigerator. Run test for 14 days

Cell preparation for bioactivity assay:

1. Assess cell density under inverted microscope.
2. Prewarm culture medium, Trypsin-EDTA, PBS in 37ºC water bath. Spray down with 70% ethanol prior to transferring to hood.
3. Remove old cell culture medium from flask. Wash cells once with PBS (5 ml), rock gently, and aspirate the PBS.
4. add 2 ml Trypsin-EDTA solution to flask and place in incubator for 3 minutes. observe cells using inverted microscope to assess detachment. This should occur within 5 – 15 minutes.
5. resuspend cells by adding 8 ml of culture medium so that a total of 10 ml of solution is contained within the flask. Rinse the sides of the flask to maximize the number of cells harvested. Count the cells using a hemacytometer
6. Inoculate 96 well plate at 2 x 10^4 cells to a total volume of 200 ul.

7. incubate plate at 37ºC and 5% CO2 for 12 hours. Verify that cells are adhered to flask bottom
8. change media.
9. Incubate plate at 37ºC and 5% CO2 for 72 hours.

ALP assay:

1. Seed 20K cells per well in a 96 well black wall clear bottom TC plate in 8% FBS media (200 uL media per well).
2. Next day aspirate media off cells and do a 1X rinse with 2% serum media.
3. Then aspirate this off and dose cells at a concentration of 1 ug/ml of BMP-2
4. Incubate treated plate for 72h at 37C w/ 5% CO2.
5. To stop plate first aspirate all the media in the wells of the plate and add 50 uL of mPERlysis buffer and freeze plate for 12 minutes at -80C and then thaw it on plate shaker (200 rpm). Once plate has thawed, prepare PNPP substrate. Prep steps listed below.

a. In a 15 mL conical tube that is wrapped in foil (PNPP is light sensitive), add 1 TBS buffer tablet and diH2O to that solution add 1 PNPP tablet.
b. Add 50 uL of this PNPP solution to each well, so the cell lysate to PNPP solution ratio is 1:1.

6. Allow reaction to proceed for 10 to 30 minutes and read absorbance at 405 nm intermittently. When absorbance is close to 1 can stop reading plate, and can discard plate. If a value of 1 or above is not reached, need to allow reaction to proceed for a longer time.

Appendix C: Calibration Curves

The following standard curves were used for the various experiments.

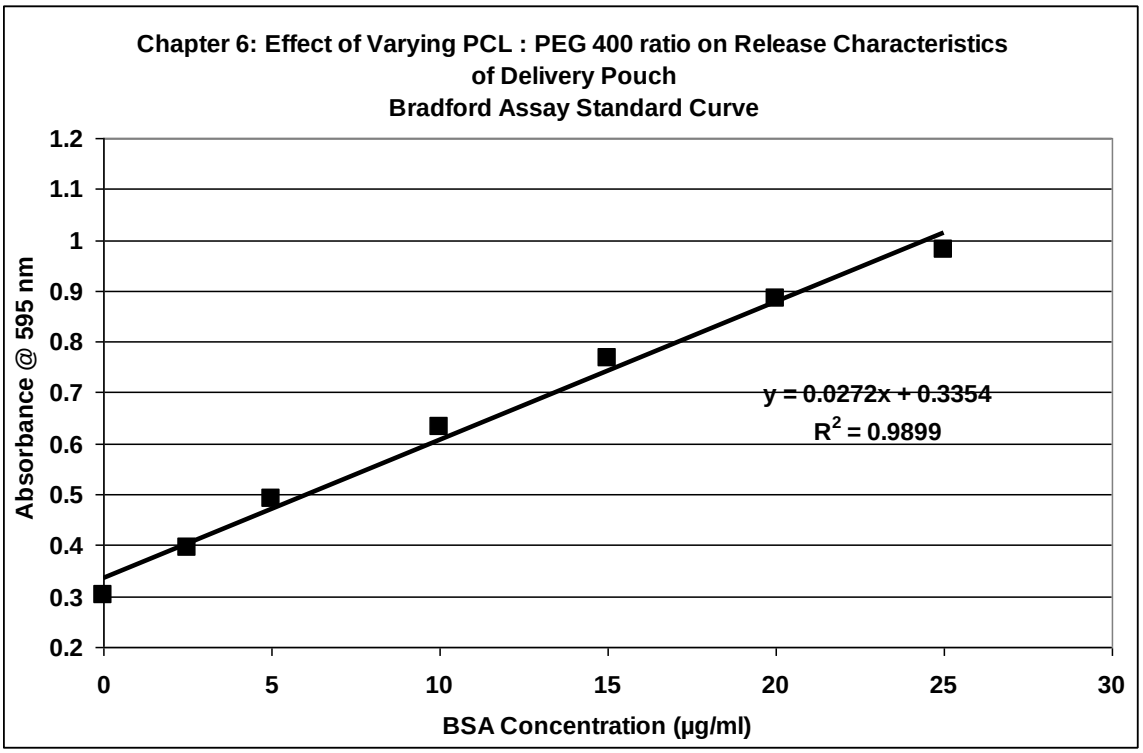

Figure A-C1: Standard Curve for BSA from 0 – 25 µg/ml, obtained via the Bradford Assay for the experiment discussed in Chapter 6: Effect of Varying PCL : PEG 400 ratio on Release Characteristic of Delivery Pouch

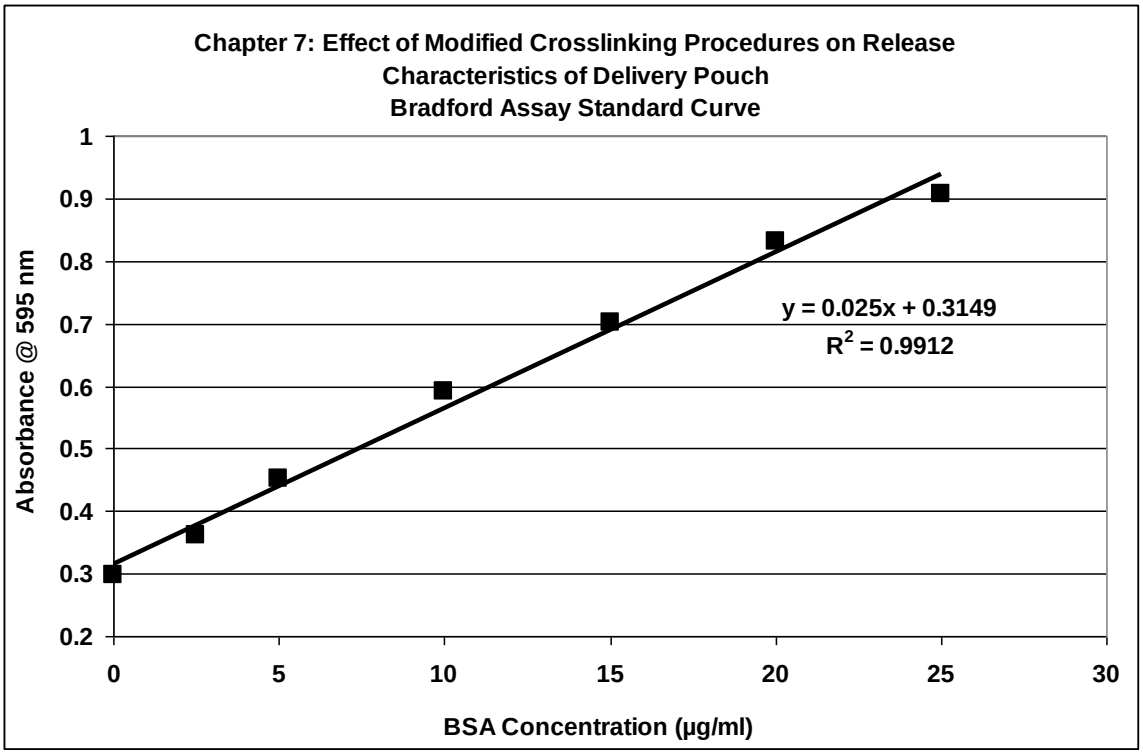

Figure A-C2: Standard Curve for BSA from 0 – 25 µg/ml obtained via the Bradford Assay for the experiment discussed in Chapter 7: Effect of Modified Crosslinking Procedures on Release Characteristics of Delivery Pouch

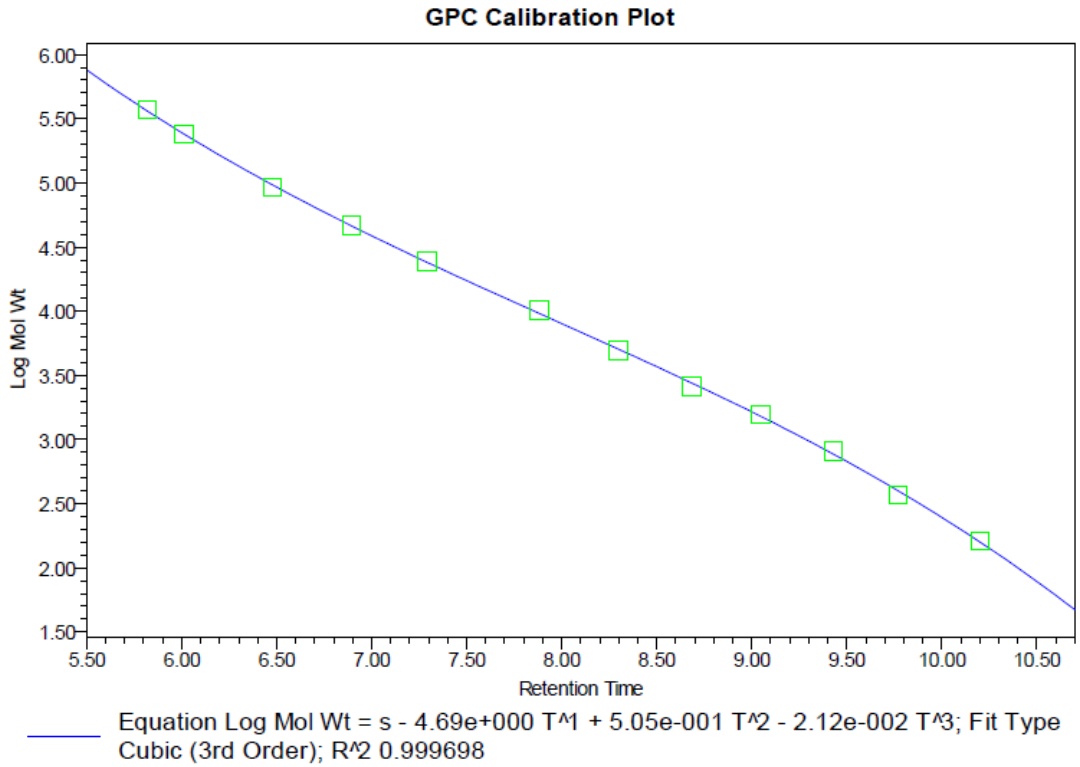

Figure A-C3: GPC Calibration Plot for Chapter 9: Characterization of Pouch Release Mechanisms with GPC.

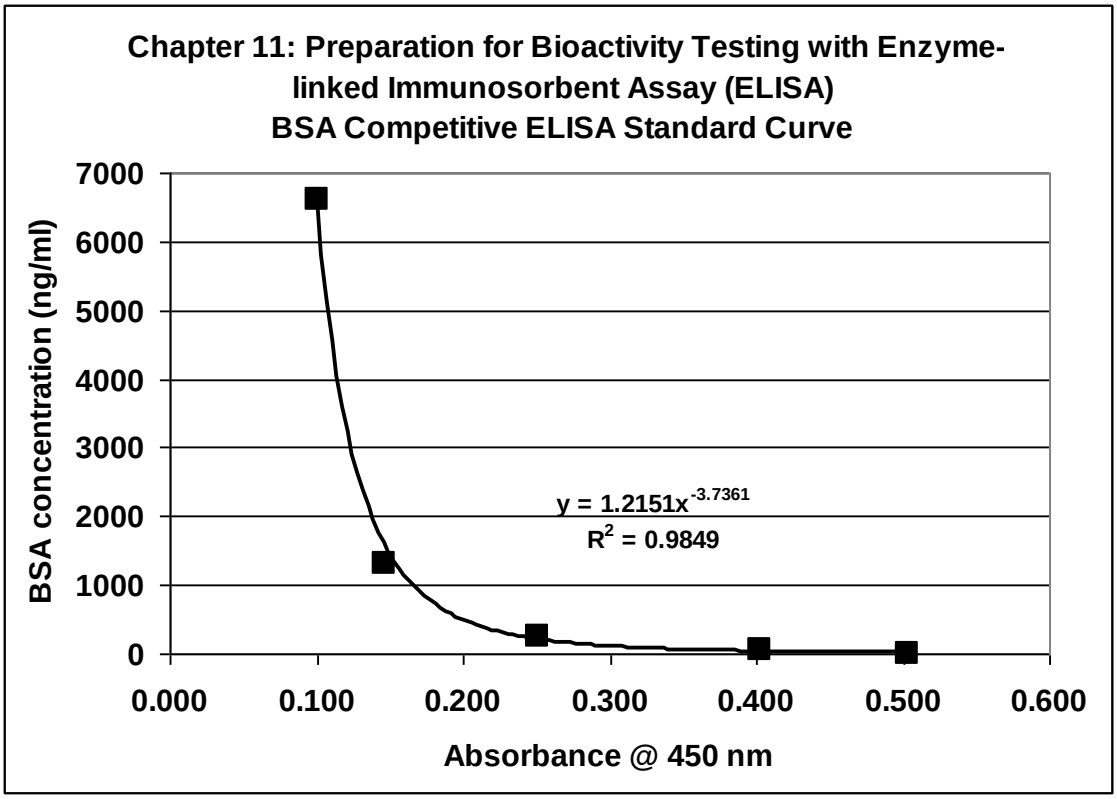

Figure A-C4: Standard Curve for BSA from 0 – 6400 µg/ml obtained via the BSA Competitive ELISA for the experiment discussed in Chapter 11: Preparation for Bioactivity Testing with Enzyme Linked Immunosorbent Assay (ELISA)

Appendix D: Equipment and Materials

Table A-D1: Lab Equipment List noting the individual pieces of lab equipment used during the project, including model names/numbers and suppliers.

Equipment	**Model Name/Number**	**Supplier**
Polypropylene Mold	7322	ThermoScientific
Vortex	Vortex Genie	VWR
Solvent Oven	Binder 9110-0099	Binder
Custom heat sealer	RES440	PACKWORLDUSA
5" x 5" polystyrene tray	89106-760	VWR
Vacuum Chamber	Lindberg BlueM V01218A	Thermoscientific
Low temperature -80ºC Freezer	Revco Ultima II	Thermoscientific
calipers	Mitutoyo CD-6 CS	Mitutoyo Corp
micrometer	Mitutoyo IP65	Mitutoyo Corp
Electronic Pipette (100-1000 µl)	PlastiBrand Transferpette® Electronic (100-1000 µl)	Cole-Parmer
Shaking water bath	Boekel Grant ORS 200	Grant Instruments
Centrifuge	Allegra 6KR	Beckman-Coulter
Type 50 Ubbelohde Viscometer	Cannon 9722-H53 (Calibrated CUSMC)	Cannon Instrument Company
GPC unit	Separation Unit 2695XE	Waters Alliance
GPC separation column	Styragel HR 4E THF 7.6 x 300 mm GPC column	Waters Alliance
GPC Calibration standards	Agilent Technologies GPC calibration standards	Agilent Technologies
UV-Vis Spectrophotometer	Cary 50BIO	Agilent Technologies
Sputter Coater	Denton Desk IV HP Sputter System	Denton Vacuum
Scanning Electron Microscope	Hitachi S570 SEM	Hitachi

Table A-D2: List of materials utilized for the project with associated catalog numbers, Grade, supplier, and Lot/Batch numbers.

Material	**Catalog Number**	**Grade**	**Supplier**	**Lot/Batch Number**
Polycaprolactone	181609-500G	research	Sigma-Aldrich	11108LE
Chloroform	372978-1L	research	Sigma-Aldrich	SHBC4115V
Sodium Alginate	W201502-1kg	research	Sigma-Aldrich	165970J
PEG 400	81172-1L	research	Sigma-Aldrich	1403539
Lyophilized Bovine Serum Albumin	SH30574.01	culture	ThermoScientific	110126100B
Calcium Chloride	C1016-100G	research	Sigma-Aldrich	070M0053V
Calcium Sulfate	237132-55/g	research	Sigma-Aldrich	MKBC9792
Ethyl Acetate	EX0245P-1	analytical	EMD Millipore	48030
PBS powder	P3813-10PAK	research	Sigma-Aldrich	SLBB5551
Bradford-Coomassie Assay kit	23236	research	Thermo scientific	NA165383
Tetrahydrofuran (THF)	TX0282-1	analytical	Sigma-Aldrich	52200
ELISA Kit for Bovine Serum Albumin	BA S-1290	research	Peninsula Laboratories	A11464